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UTILIZATION OF BARK WASTE

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UTILIZATION OF BARK WASTE

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The problem of bark waste that is generated by the forest products industry in the United States has become increasingly important. The major overall goal of the work covered in this report was to utilize physical and chemical sciences in coordinated studies to promote economic uses of bark in order to relieve pollution created by present methods of disposal. Physical utilization research included: (1) investigating the preparation of bark pellets from bark in a series of experiments designed to control several variables, such as particle size and configuration, moisture content and addition of chemical agents; (2) determining the components responsible for "self-bonding" of bark; and (3) investigating potential products from or applications of "as-is" and modified bark wastes obtained from production sources.

Chemical utilization research included: (1) preparing, for chemical studies, natural bark, bark that had been ammoniated to contain 4 percent nitrogen, and bark that had been molded into pellets and then broken down into small particles; and (2) investigating the chemical composition of each type of bark prepared.

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FOREWORD

Man and his environment must be protected from the adverse effects of pesticides, radiation, noise and other forms of pollution, and the unwise management of solid waste. Efforts to protect the environment require a focus that recognizes the interplay between the components of our physical environment--air, water, and land. The National Environmental Research Centers provide this multidisciplinary focus through programs engaged in

- studies on the effects of environmental contaminants on man and the biosphere, and
- a search for ways to prevent contamination and to recycle valuable resources.

In an attempt to solve the problem of bark waste disposal, the study described here utilized physical and chemical sciences in coordinated studies. The overall goal was to promote economic uses of bark in order to relieve pollution created by present methods of disposal.

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Director
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SUMMARY OF PROGRESS TOWARD ORIGINAL GOALS

A recent estimate of the amount of bark waste generated by the forest products industry in the entire United States totals some 14 million tons, oven-dry basis. Other estimates show six million tons are available within the west coast states of California, Oregon and Washington. Approximately half of the bark in these states now is used in some manner, leaving three million tons for which disposal problems remain. The major overall goal of the work covered in this report was to utilize physical and chemical sciences in coordinated studies to promote economic uses of bark in order to relieve pollution created by present methods of disposal. We believe substantial progress has been made toward finding economic uses for bark. Discussion of progress toward specific goals as originally outlined follows.

Physical Utilization Research

Goal 1. To investigate the preparation of bark pellets from bark in a series of experiments designed to control several variables, such as particle size and configuration, moisture content, addition of chemical agents, etc.

Progress in fulfilling Goal 1 has been very good. We have found that all species of softwood barks investigated will form acceptable pellets. This includes some eastern and northern species of bark, in addition to those from the west coast. We have found that Douglas fir bark, in particular, may be added to other types of forest industry residues (such as sawdust) in order to make a mixture which will result in formation of pellets. One significant finding was that pelletizing of bark greatly increases its bulk density and results in a product capable of being easily conveyed, stored and spread, compared to bark in natural form.

There are at least three areas where basic information resulting from our studies of bark pelletizing may be applied directly to future commercial ventures utilizing waste bark. These are as follows:

1. Pelletizing bark residues to form a fuel for production of electrical power in a centralized plant.
2. Pelletizing of bark mixed with fertilizers or other chemicals for agricultural applications.
3. Pelletizing bark to increase its bulk density and handling characteristics for easier transport of this renewable raw material.

Goal 2. One of the original goals was determination of the components responsible for "self-bonding" of bark. By a process of selective chemical extraction of Douglas fir bark followed by pelletizing (molding), it was hoped to pinpoint the compounds essential to activation of bonding (cohesion). With departure of the original Principal Investigator, this portion of the research could not be accomplished since we did not have an extractives chemist on the grant staff. We did learn that Douglas fir bark extracted by one chemical solvent system did retain its ability to form pellets and other molded items. The Forest Research Laboratory now has a separate project, F858, "Cohesion in Consolidated Bark Products" with the objective of determining the mechanism responsible for "self-bonding" of bark. We have cooperated by pelletizing bark materials.

Goal 3. To investigate potential products from or applications of "as-is" and modified bark wastes obtained from production sources.

We consider this phase of our physical utilization research efforts to be the most rewarding since it has the potential of culminating in the establishment of more than one commercial enterprise resulting directly from efforts expended in pursuing Goal 3. At this time, there are two products on the market in the area of pelletized and extruded bark; both obtained from us samples and technical information generated during the course of our studies. In addition, there is at least one other excellent possibility for the eventual establishment of a large-sized plant centered upon the chemical-physical utilization of bark wastes.

Chemical Utilization Research

Goal 1. To prepare, for chemical studies, natural bark, bark which has been ammoniated so as to contain about 4 percent nitrogen, and bark which has been molded into pellets and then broken down into small particles.

Goal 1 was completed in the 01 year of the research. The samples were then used for further chemical utilization research.

Goal 2. To investigate the chemical composition of each type of bark prepared as outlined in Goal 1.

Progress in fulfilling Goal 2 has been very good. The carbohydrate content of natural inner bark has been found to be about 50-60%. The carbohydrates have been fractionated into a soluble fraction and an insoluble "holocellulose" fraction. The ratio of sugars in the hydrolyzate from the soluble fraction has been determined as follows: glucose, 59.1; arabinose, 11.9; galactose, 3.9; mannose, 3.7; xylose, 1.0; rhamnose, 1.0. Rhamnose has not been previously reported in Douglas fir bark. The results show that the soluble fraction is composed mostly of a glucan-like polymer.

The insoluble "holocellulose" carbohydrate fraction is very easy to isolate and comprises some 35-40% of the inner bark. This fraction could become of commercial interest as a source of carbohydrates. Some suggestions for uses have been as an animal feed, and in the food industry. Hydrolysis showed the "holocellulose" to be composed of the following ratio of sugars:

glucose, 26.5; mannose, 4.1; xylose, 2.7; galactose, 1.0; arabinose, 1.1. The "holocellulose" has been further fractionated into a xylan, a glucomannan, a mannan, and a glucan.

Douglas fir bark contains an n-hexane-soluble fraction and a benzene-soluble fraction. These are called "waxes." The waxes have been studied by column chromatography, thin-layer chromatography, gas-liquid chromatography and gas-liquid chromatography combined with rapid-scan mass spectrometry. We have found sterols (β-sitosterol and campesterol) in the n-hexane wax as well as evidence for terpenes. These classes of compounds are in addition to those reported in the early literature (see text of following report). The benzene wax contains monocarboxylic fatty acids, dicarboxylic acids and hydroxy fatty acids in addition to phenolic compounds.

Whole bark was treated with gaseous ammonia to a nitrogen content of 4.08%. Extraction of the bark with organic solvents and hot water removed 1.10% of the nitrogen but did not leach out 2.98% of the nitrogen. This suggests that the ammoniation of bark may provide a material for soil amendment which possesses a quick release nitrogen supply followed by a slow-release nitrogen supply as the bark decomposes.

Bark which had been pelletized and then broken down into small particles was extracted with the organic solvents, n-hexane, benzene, ethyl ether, ethyl alcohol and hot water. These extracts were investigated by ultraviolet spectroscopy. No significant differences were noted between these extracts and similar extracts prepared from non-pelletized bark. Therefore, pelletizing does not appear to grossly alter the chemical make-up of the bark. Thus, most of the efforts of Goal 2 was directed to studies of the raw bark as described above.

Publications

1. Currier, R. A. 1971. What is bark? Physical considerations. Proceedings of conference "Converting Bark into Opportunities," Oregon State University, Corvallis, Oregon, March 8-9, 1971.

2. Currier, R. A. and W. F. Lehmann. 1971. Bark as an ingredient in molded items, particleboards, adhesives and other products. Proceedings of conference "Converting Bark into Opportunities," Oregon State University, Corvallis, Oregon, March 8-9, 1971.

3. Currier, R. A. 1972. An assessment of current bark utilization opportunities. 27th Proceedings Northwest Wood Products Clinic, Spokane, Washington.

4. Laver, M. L. 1971. What is bark? Chemical considerations. Proceedings of conference "Converting Bark into Opportunities," Oregon State University, Corvallis, Oregon, March 8-9, 1971.

5. Laver, M. L. 1971. Chemicals from bark. Proceedings of conference "Converting Bark into Opportunities," Oregon State University, Corvallis, Oregon, March 8-9, 1971.

6. Fang, H. H-L. 1971. Douglas-fir bark; n-hexane soluble fraction. Master's thesis, Oregon State University, Corvallis, Oregon.

7. Laver, M. L., H. H-L. Fang and H. Aft. 1971. The n-hexane-soluble components of Pseudotsuga menziesii bark. Phytochemistry 10(12):3292.

8. Lai, Y. C. L. 1972. Douglas-fir bark; carbohydrates solubilized by the acidified sodium chlorite delignification reaction. Master's thesis, Oregon State University, Corvallis, Oregon.

9. Laver, M. L., J. V. Zerrudo and H. Aft. 1972. Treatment of Douglas-fir bark with gaseous ammonia. Forest Products Journal 22:82.

10. Loveland, Patricia M. and M. L. Laver. 1972. Monocarboxylic and dicarboxylic acids from Pseudotsuga menziesii bark. Phytochemistry 11(1):430.

11. Loveland, Patricia M. and M. L. Laver. 1972. ω-hydroxy fatty acids and fatty alcohols from Pseudotsuga menziesii bark. Phytochemistry 11(10):3080.

12. Zerrudo, J. V. 1973. Douglas-fir bark; water-soluble carbohydrates and alkaline degradation of a xylan. Doctoral thesis. Oregon State University, Corvallis, Oregon.

Plans for Further Publications

Results of research covered by the bark pelleting studies will be presented in a paper to be published in the Forest Products Journal; title of this report will be "Pelletizing bark residues."

The research area covering bark molding appears to be appropriate for preparation of a "Technical Note" to be published in the Forest Products Journal. Plans are not finalized as yet, however.

A paper has been accepted for presentation at the IV Canadian Wood Chemistry Symposium, Quebec City, Quebec, next July. It will be published in Carbohydrate Research and cover the alkaline degradation of a xylan.

Manuscripts on "Water-soluble Carbohydrates of Bark," "Holocellulose Characteristics and Properties," "The Tannins in Douglas fir Bark" and "The Terpenes in the n-Hexane Soluble Components of Douglas-fir Bark" are in the early stages of preparation.

PHYSICAL UTILIZATION RESEARCH

I. Bark Pelleting

Using a laboratory model pelleting mill (California Pellet Mill Company, Model CL, Type 3), approximately 200 pelleting trials have been made. Raw material involved 16 species of bark plus numerous mixtures of species. In addition, runs were made on mixtures of bark plus woody residues. See Table 1 for a list of bark species and the mixtures utilized in pelleting trials.

Variables studied other than raw material mix included particle size, moisture content, fraction of bark, addition of fertilizers and seeds, pellet diameter and degree of pellet densification. Most trials consisted of 10-15 pound lots, but several weighed over 100 pounds. Various types of pellets produced are depicted in Figure 1; also shown is the hammermilled Douglas fir bark serving as standard pelletizing raw material. Pelleting concentrated on Douglas fir and western hemlock barks, since both are available in large quantities as a residue material.

Suitable pellets were formed from all species of bark studied, although problems were encountered with a few species. Where mixtures of barks or bark plus woody residues were pelleted, addition of 25% or less Douglas fir bark to the mix often spelled the difference between success and failure. Douglas fir bark always pelletized readily under a variety of conditions.

Pelleting trials were made of selected barks from the northern, eastern and southern portions of the United States in order to learn if the techniques we developed could be applied to waste barks from other regions of the nation having disposal and pollution problems. Results of our limited tests indicated bark from the species selected could be made into well-formed pellets quite readily, although the southern oak gave trouble since it was about half wood.

A potentially significant application of data collected on bark pelleting is in transportation of bark wastes. Compared to raw bark in chunk or comminuted form, the weight of pelletized bark per unit of volume occupied is 2.0 to 3.8 times as heavy. Compression factors and bulk densities for a number of bark species and pellet diameters are listed in Table 2. In addition, pelletized bark is easily conveyed, stored and spread compared to bark in natural form.

One of the most promising potential commercial applications for pelletized bark is to utilize bark as a carrier for fertilizers or other chemicals used in agriculture. During the last year of this grant, practically all the pelletizing trials were run on bark-fertilizer mixes. Small-diameter (1/8" or 3/16") pellets were made using thin dies, since this combination gave good rates of production and resulted in a product which could be spread by present standard methods of fertilizer application. Several drums of bark-fertilizer pellets were made for growing trials during the 1972 season.

Cooperation has continued with Schroeder Sales Company, Division of Pacific Kenyon Corporation, Long Beach, California, who have provided fertilizer and conducted field trials. Results of tests on a golf course in the Los Angeles area were encouraging, as were trials on home lawns in the Corvallis area and on the Forest Research Laboratory lawn. Pellets for lawn use were formulated to yield either 7.5-0-0 or 9-3-6 fertilizer values. The source of nitrogen was urea in prill form. This is a fast-acting form of nitrogen, but when mixed with bark and pelletized, the rate of nitrogen release appears to be slowed significantly. Possible economic benefits could result since slow release types of fertilizers are priced considerably higher compared to urea. Another potential source of slow release nitrogen in bark is the ammoniation process discussed in this report.

Cooperation also has continued with Bohemia Lumber Company (now Bohemia, Inc.), Eugene, Oregon. In fact, when this grant concluded on June 30, 1972, Bohemia, Inc. made a grant to us in order to continue research on a bark-fertilizer pellet, utilizing Douglas fir bark previously extracted chemically in their pilot plant. Work planned will involve production of pellets and conducting field trials on the product. Chances of constructing a commercial plant to manufacture pelletized bark-fertilizer appear very promising. A bark-plus-fertilizer pellet offers a means of complete utilization of the residue from chemically extracted bark and definitely adds to the feasibility of such an operation.

Several hundred pounds of bark-fertilizer pellets have been given to George D. Ward & Associates, Portland, Oregon, pollution control consultants. They have a contract with the Naval Facilities Engineering Command, Seattle, Washington, for reclaiming and stabilizing blow sand areas in eastern Oregon. The bark-fertilizer pellets may have the attributes of retaining moisture and slowly releasing nutrients to trees, shrubs, or grasses planted on the sand dunes.

Samples of bark pellets have been distributed widely. Included have been forest products manufacturing concerns, universities, governmental laboratories, chemical and plastics manufacturers and private individuals. A partial list of recipients of bark pellet samples may be found in Table 3.

Some limited commercial application of pelleted bark has resulted since our research work commenced. It has been learned that the largest forest products company in Canada, MacMillan Bloedel Ltd. has test marketed a bark-containing-fertilizer pellet product. The original idea appears to have come from our exploratory pelleting studies, and we have maintained contact with their research personnel.

Another company in Oregon has developed a fuel product from bark. TriWest Products of Springfield, Oregon, makes the pelletized fuel sticks. A principal of TriWest Products has consulted with us several times regarding bark pelleting, and has informed us the product idea was initiated by our research.

Trials on use of 1/2-inch diameter pelletized Douglas fir and western hemlock bark for fuel have been made by Forest Research Laboratory personnel. To date, pellets have been tried both as a stove fuel and in fireplaces. They are somewhat difficult to ignite, but once started, give off excellent heat. There has been recent discussion regarding the possibilities of using pelletized bark as the fuel source for a centrally located electric power generating plant. Instead of disposing of bark wastes through the present practices of incineration at the sawmill sites, the residues would be pelletized to improve their bulk density and shipping characteristics. Transportation to the power generator could be by means similar to the way coal is handled today. Since bark is a renewable raw material, this idea may gain more proponents as time passes.

Limited trials have been made of pelletizing bark plus seeds such as clover or grass seed. Techniques have been developed whereby the mixture can be pelletized with some degree of germination resulting. No detailed study has been made, but this is an area we hope to investigate soon.

Another use of bark in pellet form is being investigated by the U.S. Forest Service, Forest Products Laboratory, Madison, Wisconsin. Some of our 1/4-inch Douglas fir bark pellets have been used as the large aggregate in cement-bark building blocks. Tests are continuing on the process and resulting blocks.

II. Bark Molding

Much of the research on molding of bark has consisted of demonstration-type projects in cooperation with various segments of the forest products, adhesives, and plastics industry. This method of attacking the problem was utilized in order to probe the potentials of various end markets for comminuted Douglas fir and western hemlock barks and to increase awareness that such material was available from bark residues. A brief description of the various investigations follows.

A. Planter block from bark

1. Using compression molding, trays, each consisting of six planter blocks, were prepared from western hemlock bark. In the preparation of these blocks, the following variables were studied: moisture content of the bark, binder use, and addition of other materials such as lime, clay, chemical fertilizer (Vigoro) and fungicides. Greenhouse studies, followed by field planting, indicated that the bark planter blocks could grow tomatoes and pansies from seed.

2. Douglas fir and red alder barks were extruded into blocks and limited growth trials were made utilizing tomatoes. Blocks were formed with and without added urea-formaldehyde binder; chemical fertilizer (Vigoro) was added to all barks prior to forming.

B. Bark added to plastics

1. One investigation involved a three-way cooperative study with industry. We prepared dry comminuted bark, a lumber company (Dant and

Russell, Inc., Portland, Oregon) provided the raw bark and financed experimental time on molding machines at a plastics producer (Grant and Roth Plastics, Inc., Hillsboro, Oregon). Several types of extruded, sheet formed and injection molded products have been produced, with bark extension of the plastic in the order to 40-60 percent. Types of plastics extended by bark include polyethylene, cellulose butyrate, cellulose acetate, polyurethane, polystyrene and polyvinyl chloride. Photographs showing some experimental products may be found in Figure 2.

2. The plastics company involved in study (1) above (Grant and Roth Plastics) sent some of the bark extender to Eastman Chemicals, Inc., Kingsport, Tennessee, for their evaluation. A laboratory report indicated promise for a cellulose acetate + plasticizers + bark mix. Cost per pound of this mix was estimated to be lower than polyvinyl chloride.

3. Large samples (60 lb) of -20 mesh Douglas fir and western hemlock bark were shipped to Borden, Inc., Thermoplastic Division, Leominster, Massachusetts, where they were evaluated as fillers for extruded rigid PVC pipe. The resulting laboratory report by Borden, Inc. indicated the bark contained an excessive amount of volatiles, leading to porous and lumpy extrusions. On the positive side, addition of bark lowered specific gravity of the product, resulting in a cost-volume advantage. A follow-up study now is underway whereby Borden, Inc. has been provided another sample of Douglas fir bark which has had some potential volatiles removed by chemical extraction. No results have been received to date.

4. The Hysol Divison of the Dexter Corp., Industry, California, has formulated a knothole or other void filling compound from -20 mesh Douglas fir bark plus epoxy plastic materials. The compound has been used commercially to fill voids in rough-sawn textured plywood panels.

We have provided over 100 lb of comminuted bark for these experimental formulations, which have resulted in commercial production of products numbered AE1502 and AE1504.

5. Preliminary runs have been made extruding venetian blind slats from polystyrene plus -20 mesh Douglas fir bark in both plain and extracted form. This experimentation was sponsored by Omega Industries of Salem, Oregon. Five different color slats have been run, with promising results. The extracted bark appeared to be the more suitable extender.

6. During the last few weeks of the grant period, an excellent contact was made with B. F. Goodrich Chemical Company, Avon Lake, Ohio. This company is one of the largest suppliers of plastics molding compounds. Large samples of both unextracted and extracted Douglas fir bark, and bast fibers have been shipped for trial runs on soil pipe, conduit and etc. No results have been received yet.

7. A few furniture drawer guides were made from 50% -20 mesh Douglas fir bark and 50% general purpose polystyrene at Modcom, Inc., Canby, Oregon. The management there is willing to conduct more evaluation tests when some free plastic machine time is available.

C. Other molded products from bark

1. Molded bark products not involving plastics have resulted in cooperative efforts with two different commercial companies interested in production of extruded "fuel logs" from bark wastes. A sample lot of Douglas fir bark was prepared for Glomera-American, Inc., Reedsport, Oregon, who shipped it to Switzerland where the log forming machines are made. Trail runs indicated a log containing mostly bark can be formed by this process. Another company, Oregon Timber Products Company, Albany, Oregon,

has purchased log extrusion machines from Japan; we cooperated with them in developing a log containing substantial quantities of Douglas fir and western hemlock barks. Unfortunately, this company has suffered financial problems and is not operating at the present time. Photographs depicting these various logs may be found in Figure 2.

III. Other Bark Products or Uses

Samples of bark or bark fractions have been prepared for a number of other companies and researchers. Some examples follow.

1. Bark particles have been prepared for experimental work on a trickling filter system for disposal of animal wastes. This is being done by Dr. Myron Cropsey, Agricultural Engineering Department, Oregon State University. We have provided all of the 3/4- to 3-inch Douglas fir bark chunks used in his research. Dr. Cropsey presented a paper covering his results to date at a meeting of the American Society of Agricultural Engineers in Portland, Oregon, October 8, 1971. His tests will continue during the 1972-1973 academic year.

2. Bohemia, Inc., Eugene, Oregon, is interested in developing a process for extraction of wax from Douglas fir bark, and has built a pilot plant. After wax extraction, over 90% of the bark still remains; we have cooperated with Bohemia, Inc. in determining potential uses for this spent bark. One effort has been to turn the residue into an extender for phenolic plywood glues. Extracted bark was shipped to us and we ground and screened over 400 lbs to prepare -100 mesh material. This was successfully evaluated as an extender for plywood glues, undergoing standard test procedures prescribed by the American Plywood Association. Several of the chemical companies manufacturing plywood glues are interested

in the bark extender. Cooperation is continuing with Bohemia, Inc. in separating the physical components (cork and fiber) of extracted Douglas fir bark prior to grinding the remaining portion as an extender. Possibilities of commercialization of the process appear encouraging.

3. Samples of the -100 mesh extracted bark extender mentioned in (2) above, as well as -100 mesh unextracted whole bark have been sent to Colorado State University, where a graduate student is evaluating their possible use as an extender for binders currently used in manufacture of particleboard. Preliminary results look promising; additional bark has been requested. No published report is available as yet.

IV. Miscellaneous

During the period January 1, to June 30, 1972, the physical utilization research done under this grant was presented orally at four meetings as follows.

1. Western Builders Short Course, Portland, Oregon. February 11, 1972.

2. Twenty-seventh Annual Northwest Wood Products Clinic meeting jointly with the Inland Empire Section of the Forest Products Research Society. Spokane, Washington. April 17-19, 1972.

3. Pacific Northwest Section of the Forest Products Research Society, Vancouver, B. C., Canada. May 9, 1972.

4. Twenty-sixth Annual Meeting, Forest Products Research Society, Dallas, Texas. June 19-22, 1972.

In addition, a report for local radio broadcast also was made; this covered both physical and chemical research sponsered by this grant.

CHEMICAL UTILIZATION RESEARCH

INTRODUCTION

There are a number of general review articles on bark utilization, but three pertinent ones are by Harkin and Rowe (1), the Bark Committee of the Forest Products Research Society (2) and J. Alfred Hall (3). Hall comments that one cannot write a meaningful "Chemistry of Douglas-fir Bark" in the present state of our knowledge. This statement demonstrates the great gaps in the knowledge of the chemistry of bark. The research herein reported is an effort designed to provide a better understanding of the chemistry of Douglas-fir bark with the goal of a more complete and better utilization of this abundant natural raw material.

The work has been organized into three areas: the carbohydrates; the waxes; and the ammoniation of whole bark. For clarity of reporting, each of these subjects is presented separately. Each section includes an historical review, an experimental, and a results and discussion.

BARK CARBOHYDRATES

I. Historical Review

Authors have referred to Douglas-fir as *Pseudotsuga taxifolia* (Poir.) Britt. and other names. However, the presently preferred botanical name is *Pseudotsuga menziesii* (Mirb.) Franco. The names all refer to the same genus and species. Mention is made of this to avoid confusion about the exact species investigated.

The present work is concerned with Douglas-fir inner bark. This is a specific anatomical part of the bark and a brief description of bark anatomy is included for purposes of definition.

For a detailed anatomical description of Douglas-fir bark, reference is made to Grillos (4), Grillos and Smith (5), Chang (6), and Ross and Krahmer (7). Briefly, however, bark can be considered to consist of inner bark and outer bark (Figure 4). The inner bark (phloem cells) is the portion from the vascular cambium to the innermost cork layer. The outer bark (rhytidome) is everything to the outside of the innermost cork cambium (Figure 4).

The inner bark comes from the vascular cambium, that layer of living cells between the wood and bark which divide to form wood to the inside and bark to the outside. The inner bark is composed mainly of sieve cells, axial and ray parenchyma, and sclereids (Figure 4). Much of the inner bark is living in the living tree because many of the parenchyma and sieve cells remain alive as long as they are components of the inner bark.

Douglas-fir sclereids are short, sharply pointed, spindle-shaped fibers of a red brown color (Figure 4). They are often referred to as bast fibers. They are lignified cells and develop from axial parenchyma cells some distance from the vascular cambium. In becoming sclereids, axial parenchyma cells approximately 0.1 to 0.5 mm in length elongate to 1 to 2 mm by apical intrusive growth, and form thick walls. The sclereids are commonly straight and somewhat cigar-shaped. Kiefer and Kurth (8) and Ross and Krahmer (7) describe and illustrate the general appearance and position of the sclereids in Douglas-fir bark.

The outer bark of Douglas-fir consists of layers of cork in which growth increments are usually visible, as shown by Ross and Krahmer (7). Interspersed among the corky layers are areas of phloem tissue that contain the sclereids and other cell types found in the inner bark (Figure 4).

The cork layers form from the cork cambia which are living cells that were once living parenchyma cells of the inner bark. New cork cambia form in the inner bark and cut away part of the inner bark, which now becomes part of the outer bark. The cork cambium produces cork cells to the outside and a few storage cells to the inside. Cork cells have thin cellulose walls which are coated with suberin. Suberin is essentially an ester condensation polymer of hydroxylated, saturated and unsaturated straight-chain fatty acids (9). The cork layers may also contain tannin, dihydroquercetin and starch. All cells outside the innermost cork cambium are dead because no food supply can pass through this layer of cork cells. This then results in an outer bark composed of cork cells and dead phloem cells, which were once inner bark.

The studies on the anatomy of bark have shown it to be a complex physical material. The chemical composition of bark is equally complex. It is known, however, that the major constituents of bark are the carbohydrates, just as they are the major constituents in wood (9, 10). However, little attention has been devoted to the carbohydrates present in the bark of trees. In contrast, the carbohydrates in the wood of trees have been extensively studied. Polysaccharides such as pectinic acids, galacturonogalactans, arabinogalactans, 4-O-methylglucuronoxylans, arabino-4-O-methylglucuronoxylans, glucomannans, galactoglucomannans, and cellulose have been isolated from the wood of numerous species of both gymnosperms and arborescent angiosperms and their chemical structures elucidated (11, 12, 13, 14, 15 p. 447)

One reason for this has undoubtedly been the greater economic importance of wood as compared with bark. Another is probably to be found in the occurrence in bark of non-carbohydrate constituents such as suberin, tannins, phlobaphenes, and various phenolic compounds, all of which make the isolation of polysaccharides from bark difficult. Wood contains none or few of these components.

Segall and Purves (9) discussed these difficulties in a review of the early literature on the chemistry of bark. The so-called "extractives" were known to interfere unless they were removed by exhaustive, successive extractions with alcohol, water or other neutral, chemically inert liquids, such as diethyl ether or petroleum ether. This early literature showed that after all of the solvent-soluble materials had been removed by exhaustive extraction, some 70 to 90% remained as an undissolved residue.

After removing the extractive materials it was possible to acid hydrolyze the remaining residue and investigate some of the monosaccharides released. It appeared that the polysaccharides in bark were based predominantly upon glucose, and to a lesser extent on galactose, mannose, xylose, arabinose and rhamnose. The existence of true cellulose could not be established at that time.

Kurth (10) also reviewed the early literature on the chemical composition of barks. He reported that barks contained hemicelluloses and "cellulose"; the sugars associated with the hemicelluloses were glucose, galactose, mannose, arabinose, and xylose. The "cellulose" was simply that fraction which remained after treating extracted bark with four successive portions of a mixture of one part nitric acid and four parts alcohol. No additional evidence that the material was cellulose was given.

Kiefer and Kurth (8) isolated a holocellulose fraction from the bast fibers, or sclereids, of Douglas-fir bark. Whole bark was collected, ground and screened. All fractions larger than 40-mesh were reground and rescreened. The fibers were obtained in an almost pure state by stirring the crude fiber fraction in five times its volume of distilled water at room temperature. By virtue of their high specific gravity, the fibers readily sank to the bottom of the container, whereas cork and other impurities remained on the surface and were skimmed off.

The overall percentage composition of the bast fibers was determined and is presented in Table 4. For sake of comparison, analyses of Douglas-fir wood, taken from the literature (16), are included in the table. The data indicate that the bast fibers may be a lignocellulose material with a composition similar to that of wood. The higher lignin content of the bast fibers and the lower methoxyl content of this lignin, however, are notably dissimilar from those found in wood.

Paper chromatograms of an acid hydrolyzate of the holocellulose showed the presence of glucose, galactose, mannose and xylose. The limited techniques of paper chromatography at that time did not resolve mannose and arabinose. Their fermentation procedures for quantitative analyses indicated an apparent absence of arabinose in Douglas-fir bark. This paper by Keifer and Kurth (8) and the one by Holmes and Kurth (17) appear to be the major works published on the carbohydrates in Douglas-fir bark. These materials seem to have been largely ignored to date.

II. Experimental

A. Collection of Bark Samples

On May 22, 1969, the outer bark was chipped from a standing Douglas-fir of diameter 18.8 inches at breast height at Black Rock, Oregon. The inner bark plus cambium were then carefully stripped from the tree and immediately brought to the laboratory where the cambium layer was removed.

The cambium-free inner bark (4832.0 g, moisture content 44.9%, hot air oven at 110°C) was immersed in 18 liters of 95% ethanol. Water (1207.0 ml) was later added to adjust the solution to ethanol-water (4:1 v/v) with calculations for the moisture content of the inner bark.

The tree was later cut and by count of the annual rings was 130 years old.

B. Sample Preparation

The inner bark, after soaking in the ethanol-water (4:1 v/v) for three days, was recovered by filtration and washed well with fresh ethanol-water (4:1 v/v).

The combined filtrate and wash liquors were concentrated on a rotary evaporator (Büchi, Rotavapor, Switzerland) to less than 2 liters. Water was added to exactly 2 liters in a volumetric flask and three 10-ml aliquots of the adjusted solution were removed and the nonvolatile solids determined by the Celite-vacuum drying method (18). The values obtained were 2.057 g per 10-ml aliquot which corresponded to a total of 411.4 g of material in the ethanol-water (4:1 v/v) extract or 15.4% of the original inner bark on a dry weight basis.

The extract was tested for monosaccharides by paper chromatography using the solvent system ethyl acetate-pyridine-water (8:2:1 v/v/v). A trace of glucose was detected by spraying the chromatograms with o-aminodiphenyl reagent (0.4 g o-aminodiphenyl dissolved in a solution prepared from 100 ml of glacial acetic acid and 20 ml of distilled water) and heating at 100±2° in an oven for 5 min. (19).

The residue of inner bark (air-dried) was ground in a Wiley Mill (A. H. Thomas Company, Philadelphia, PA) and fractionated according to particle size by screening with screens of an increasing number of meshes per inch

(The W. S. Tyler Company, Cleveland, Ohio). All fractions were examined under a microscope. It was observed that those materials retained on the screens of 20 and 35 meshes per inch were bundles of fibers. These fractions were reground and rescreened. All materials (1612.8 g) which passed through 35, 60, and 80 mesh per inch screens and were retained on a 100 mesh per inch screen were used in preparing the sample for future investigations. In addition 238.3 g out of the 390.0 g which passed through a 100 mesh per inch screen but were retained on a 150 mesh per inch screen were included. None of the larger material (23.6 g) retained on the 35 mesh per inch screen was used.

C. Benzene-Ethanol Extraction

A part of the recombined bark fractions (1500.0 g, dry weight) was divided into three portions (618.0 g, 618.0 g, and 264.0 g dry weight). Each portion was extracted with a solution of benzene-ethanol (2:1 v/v; 1400 ml of benzene and 700 ml of ethanol for the large portions, and 598 ml of benzene and 299 ml of ethanol for the small portion) in a Soxhlet extractor. Each extraction was continued for 37.5 hr (a minimum of 50 solvent exchanges).

The non-volatile solids in the benzene-ethanol extract were determined by the Celit-vacuum drying method (18); weight, 77.9 g or 4.4% of the original inner bark on a dry weight basis.

The extract was tested for monosaccharides by paper chromatography using the solvent system ethyl acetate-pyridine-water (8:2:1 v/v/v). No sugars were detected by the o-aminodiphenyl spray reagent described earlier.

D. Hot-Water Extraction

A part (1458.3 g, dry weight) of the air-dried residue remaining from the benzene-ethanol (2:1 v/v) extraction was divided into four portions (three of 363.8 g and one of 366.9 g, dry weight). Each portion was stirred into 3 liters of distilled water at 55° in a 4-liter beaker. The extraction was continued at 50°-60° for 24 hr with intermittent stirring.

The mixture was separated by filtration using a Büchner funnel. The filtrate was condensed on a rotary evaporator, freeze-dried and the solids weighed; weight, 202.5 g or 11.1% of the original inner bark on a dry weight basis.

E. Ammonium-Oxalate Extraction

A part (1458.3 g, dry weight) of the residue (dried in a hot-dry room at 32° and relative humidity 31% for 4 days) was divided into 4 fractions each containing 312.0 g. Each fraction was extracted with 3 liters of 0.5% aqueous ammonium oxalate in a 4-liter beaker at 70-80° for 26 hr.

The insolubles were recovered on a Büchner funnel, washed thoroughly with water and dried in a hot-dry room at 32° for 3 days; weight 1198.0 g or 66.3% of the original inner bark on a dry weight basis.

The filtrate was concentrated on a rotary evaporator, freeze-dried, and the solids (3.5 g air-dry weight) were stored for future reference.

F. Acidified Sodium Chlorite Delignification; Isolation of a Holocellulose Fraction

An amount (1194.0 g dry weight basis) of the above residues from the ammonium oxalate extraction was divided into six batches. Five batches contained 196.8 g each and one contained 210.0 g. Each batch was stirred into 3.0 liters of distilled water at 75-80° and the temperature was maintained throughout the reaction. A steady stream of nitrogen was bubbled through the mixture to prevent the accumulation of gases and the mixture was stirred mechanically. Glacial acetic acid (20.0 ml) was added, followed by sodium chlorite (60.0 g). Fresh glacial acetic acid and sodium chlorite were added two more times at one hour intervals. During the second addition foaming occurred on top of the beaker so 2-5 ml of isoamyl alcohol was added into the foaming mixture to prevent additional foaming. At the end of 4 hours, the yellow solids were recoverd by filtration using a Büchner funnel with Whatman No. 1 filter paper, and washed with distilled water. The yellow solids were dialyzed for one week, washed with distilled water, dried with ethanol and finally dried in the air for two weeks; weight 797.6 g, dry weight basis or 44.3% of the original inner bark. The filtrate was dialyzed for one week, concentrated on a rotary evaporator and the solids recovered by freeze-drying. They were stored for future reference (see section II-H).

The yellow color of the residue from the acidified sodium chlorite treatment indicated incomplete delignification. Therefore, the delignification was repeated on 783.9 g (dry weight basis) of the yellow solids (20). The residue was recovered by filtration, dialyzed for one week and freeze-dried. The white-colored, acidified sodium chlorite insoluble, solids which were thus obtained were termed "holocellulose"; weight 540.9 g (dry weight) or 30.6% of the original inner bark on a dry weight basis. The holocellulose was pure white and retained some of the fiber-like characteristics of the starting material (Figure 3).

The filtrate from the second delignification reaction was not investigated further.

G. Characterization of the Hot-Water-Soluble Solids

(Isolation described in section II-D)

1. Elemental Analyses for Nitrogen, Sulfur, Phosphorus, and the Halogens

A small piece of freshly cut sodium was wiped thoroughly to remove all traces of kerosene and placed in a small glass test tube. The tube was gently heated in a flame until the sodium melted and the vapors rose 1-2 cm up the walls of the tube. A small amount of the hot-water-soluble solids was added to the molten sodium and the tube was heated strongly over an open flame. Heating was continued for 1 to 2 minutes. After the entire end of the tube was red hot, the tube was plunged into an evaporating dish which contained about 10.0 ml of distilled water. The hot end of the tube shattered and the resulting mixture was heated to boiling, the insolubles removed by filtration, and the filtrate recovered for elemental analyses.

An aliquot (2.0-3.0 ml) of the filtrate was added to powdered ferrous sulfate (0.1 g) in a test tube. The solution was heated gently with shaking until it boiled. Sufficient dilute sulfuric acid was added to dissolve the iron hydroxides and to give an acid solution. A precipitate of Prussian blue formed, which indicated the presencv of nitrogen. For purposes of comparison, alanine was fused with sodium, and tested for nitrogen. A Prussian blue color formed which showed the presence of nitrogen.

A second aliquot (2.0 ml) of the filtrate was acidified with dilute acetic acid, and a few drops of lead acetate were added. No precipitate formed, indicating that sulfur was not present. As a control for the analysis of sulfur, cystine was fused with sodium and analyzed exactly as described above. A yellow precipitate formed which confirmed the presence of sulfur.

A third aliquot (1.0 ml) of the filtrate was acidified with concentrated nitric acid (3.0 ml) and boiled for 1 minute. The solution was cooled and an equal volume of ammonium molybdate reagent was added. The solution was warmed to 40-50° and allowed to stand. No precipitate formed, indicating that phosphorus was not present. Glucose-1-phosphate was fused with sodium and tested for phosphorus. A yellow precipitate (ammonium phosphomolybdate) formed which indicated the presence of phosphorus.

A fourth aliquot (2.0 ml) of the filtrate was acidified with dilute sulfuric acid, boiled gently to remove any hydrogen cyanide which might be present, and the solution was treated with a few drops of aqueous silver nitrate. No precipitate formed indicating that none of the halogens were present.

A small sample of the hot-water-soluble solids was quantitatively analyzed for nitrogen (Kjeldahl, 3.63%; Pascher and Pascher, 53 Bonn, Buschstrasse 54, West Germany).

2. Test for Tannins and Starch

A small amount of the hot-water-soluble solids was dissolved in distilled water. An aliquot (1.0 ml) of this solution was treated with a few drops of a ferric chloride-potassium ferricyanide solution (21, p. 227) (1% solutions are mixed prior to use). A blue color developed which indicated the presence of phenolics.

A second aliquot (5.0 ml) of the solution was treated with a few drops of iodine indicator. A deep blue color developed which indicated the presence of starch.

3. Strong Acid Hydrolysis

A portion of the hot-water soluble solids (0.07 g) was treated with 72% sulfuric acid (0.9 g) and allowed to stand for 45 minutes at room temperature. Water (20.1 ml) was added slowly with stirring to provide a final concentration of 3.0% acid. The solution was refluxed for 5 hours, cooled to room temperature, and neutralized to pH 5.0 with saturated aqueous barium hydroxide solution. The resulting barium sulfate precipitate was removed by centrifuge, washed well with water and the washings were added to the decantate. The combined decantate was concentrated under vacuum on a rotary evaporator to about 50.0 ml.

4. Mild Acid Hydrolysis

A portion of the hot-water-soluble solids (0.32 g) was dissolved in 3.0% sulfuric acid (96.0 ml) and the solution refluxed for 5 hours. After cooling to room temperature, the solution was neutralized to pH 5.0 with saturated aqueous barium hydroxide solution. The precipitate of barium sulfate was removed by centrifuge and washed with water. The decantate plus the washings were concentrated to about 100.0 ml under vacuum on a rotary evaporator (22).

5. Qualitative Amino Acid Analysis by Paper Chromatography

The hydrolyzates from the mild acid hydrolysis were subjected to ascending two-dimensional paper chromatography (23, 24, p. 93) on Whatman No. 1 paper, using water-saturated phenol as a developer in one direction in an atmosphere of ammonia, and n-butanol-formic acid-water (20:6:5 v/v/v) as developer in the second direction. A solution of ninhydrin (1.0 g) dissolved in n-butanol (500.0 ml) was used as the spray reagent.

6. Carbohydrate Analysis by Paper Chromatography

A portion of the hot-water-soluble solids was dissolved in distilled water, spotted on Whatman No. 1 filter paper and paper chromatographed as described below to determine if free sugars were present. No free sugars were detected.

The hydrolyzates from the strong acid hydrolysis and the mild acid hydrolysis were applied at intervals of about 1 inch along one edge of a Whatman No. 1 filter paper, 18 x 22.5 inches, in such amounts as to produce a colored spot easily detectable with the naked eye in a natural light or under ultraviolet light. A standard solution containing about 1.0% each of the known monosaccharides, glucose, mannose, galactose, arabinose, xylose, and rhamnose was also spotted on the filter paper. The sugars were separated by descending development with ethyl acetate-pyridine-water (8:2:1 v/v/v) (25) as developer. The solvent was allowed to migrate almost to the bottom of the papers at which time they were removed from the tank and air-dried for at least 6 hours. Some of the papers were returned to the tank and developed as before (repeated up to 3 or 4 times) in order to obtain a better separation.

The paper chromatograms were sprayed with o̲-aminodiphenyl reagent (0.4 g o̲-aminodiphenyl dissolved in 100.0 ml of glacial acetic acid and 20.0 ml of distilled water) and heated at 100±2° in an oven for 5 minutes (26, 19). The spots were outlined in pencil under ultraviolet light. The rates of movement of the hydrolyzate sugars were compared with those of authentic samples when run simultaneously on the same chromatograms.

The o̲-aminodiphenyl was purified by recrystallizing the technical grade material twice from aqueous ethanol. Activated charcoal was used as a decolorant. The purified crystals were dried at room temperature under vacuum.

7. Purification of the Hot-Water-Soluble Solids

A portion of the hot-water-soluble solids (3.0-4.0 g) was stirred into a small amount of distilled water until it was thoroughly wetted. Distilled water at room temperature was then added slowly with stirring until the mixture had a concentration of 1.0%. Stirring was continued for at least 2 hours at room temperature. The mixture was allowed to stand and was then centrifuged. The supernatant liquid was recovered. The undissolved solids were again stirred into water and the whole process repeated 3 more times. The liquor from the fourth washing was colorless and clear.

The undissolved solids which remained were freeze-dried; yield 29.8% of the original hot-water-soluble solids. These were labeled "Fraction A."

A portion of "Fraction A" was tested for starch (positive) and tannins (negative). A second portion of "Fraction A" was hydrolyzed by strong acid, and the hydrolyzate was examined on paper chromatograms. The chromatograms showed glucose only. A large sample of "Fraction A" was later prepared for additional research purposes.

All of the liquors from the above treatments were combined and concentrated to about 1.0 liter under vacuum at less than 30° temperature in a rotary evaporator. Ethanol (95%) was added to provide a final solution of 70.0% ethanol (1.0 liter each time). After the fourth washing, the precipitate was dissolved in water and traces of ethanol were removed under vacuum in the rotary evaporator. The polysaccharide was then recovered by freeze-drying and labeled "Fraction B"; yield 25.9% of the original hot-water-soluble solids. A portion was tested for starch (positive) and tannins (faintly positive). Another portion was hydrolyzed under mild acid conditions, and examined by paper chromatography. The chromatograms showed the presence of amino acids and sugars.

The mother liquor and washings from "Fraction B" were combined, concentrated, and freeze-dried. This sample was labeled "Fraction C". The yield of "Fraction C" (by difference) was 44.3% of the original hot-water-soluble solids. A portion of this sample was tested for starch (negative) and tannins (positive).

8. Enzyme Hydrolysis to Remove Starch

The enzymes used were two commercial preparations purchased from Marschall Division, Miles Laboratories, Elkhart, Indiana. The first enzyme, HT-1000 (27) is a mixture of amylolytic and proteolytic enzymes, capable of faster and more economical liquifactions of starch than many other α-amylases. It has been derived from *Bacillus subtiles*, and is in the form of a white, dry powder. The second enzyme (commercial name, Diazyme L 30) is an amyloglucosidase (28). It is sold in liquid form.

A part (15.0 g) of "Fraction B" was dissolved in distilled water (85.0 ml) and the pH was adjusted to 5.5-7.0 with sodium carbonate solution (1.0 N). HT-1000 (7.5 mg, 0.05% based on sample weight) dissolved in a small amount of distilled water was added to the sample solution. The mixture was heated to 75° in a water bath with continuous agitation and held at that temperature for 15 minutes. Heating was then continued until the temperature reached 85-87° and held at this level for 30-40 minutes. At the end of this time, the sample and water bath were cooled to 60°, and the pH of the sample was adjusted to 3.8-4.2 with hydrochloric acid (0.1 N). Diazyme (0.09 ml, equivalent to 80 units/lb starch) was added directly to the cooled carbohydrate solution. The mixture was incubated at 60° with stirring for 72 to 96 hours. At the end of the incubation period, the mixture was transferred to a dialysis bag and dialyzed for two days in distilled water, one week in running tap water and another day in distilled water. The non-dialyzable portion was concentrated in a rotary evaporator at less than 40° and the concentrate was freeze-dried; yield 9.9% of the original hot-water-soluble solids.

The materials passing out of the dialysis bag during the first two days were collected, concentrated, and freeze-dried. A portion of the freeze-dried sample was dissolved in water, spotted on Whatman No. 1 filter paper and paper chromatographed. The chromatograms showed glucose only. Another portion of the sample was hydrolyzed under mild acid conditions and the hydrolyzate was tested by paper chromatography. The chromatograms showed glucose only.

9. Enzyme Hydrolysis to Remove Protein

The non-dialyzable, freeze-dried material recovered from the enzyme hydrolysis to remove starch was dissolved in 1.5 liters of distilled water. Tris (hydroxymethylamino)-methane was added to the solution and the pH was adjusted to 8.5 with 0.1 N hydrochloric acid. Chymotrypsin (160 mg) and trypsin (150 mg), dissolved in a small amount of water, were added to the buffered solution and the volume adjusted to 2.0 liters. The solution was placed in a hot-water bath and heated at a constant temperature of 30-40° with constant stirring for 12 days. At the end of the incubation period, the solution was transferred to a dialysis bag and dialyzed for 12 days against running tap water. The precipitate which had formed during the reaction was separated from the mother liquor by centrifugation and washed three times with distilled water. The mother liquor and the washings were combined, concentrated in a rotary evaporator and freeze-dried (29). The freeze-dried material had a tan, fluffy appearance. This sample was labeled "Fraction D"; yield 1.5% of the original hot-water-soluble solids. "Fraction D" was hydrolyzed with 3.0% sulfuric acid and investigated by paper chromatography. The chromatograms showed the presence of galactose, glucose, arabinose and traces of rhamnose, xylose, and mannose.

10. Carbohydrate Analysis by Gas-Liquid-Chromatography

The gas-chromatograph used was a Hewlett-Packard 5751B Research Chromatograph (Hewlett-Packard Company, Palo Alto, California) equipped with dual flame ionization detectors. The conditions were: column, 6.5% ECNSS-M on Gas Chrom Q 100/120 mesh, 6 ft x 1/8 in. O.D. stainless steel; injection port 200°, detector 235°; column temperature 175° isothermal; helium flow 30 ml/min; range setting 10^2; attenuation setting 16.

The various samples were hydrolyzed and the derivatives prepared for injection into the gas chromatograph as follows. The polysaccharide sample (0.32 g) was dissolved in 3.0% sulfuric acid (96.0 ml) and refluxed for 5 hours (mild acid hydrolysis). The solution was cooled and authentic myo-inositol (0.1000 g) was added. The hydrolyzate was then neutralized to pH 5.0 with a saturated aqueous barium hydroxide solution. The resulting barium sulfate precipitate was removed by centrifuge. An aliquot (25.0 ml) of the clear supernatant solution was transferred to a round-bottomed flask (100.0 ml). Sodium borohydride (0.08 g) was added to the flask and allowed to react for 2 hours at room temperature (30, 31).

The excess sodium borohydride was decomposed by adding acetic acid until gas evolution ceased. The solution was concentrated to a sirup in a rotary evaporator, and methanol (10.0 ml) was added and re-evaporated. The addition and removal of methanol was repeated five times (32). The resulting sirup was dried in an oven at 105° for 15 minutes to ensure complete removal of water.

Acetic anhydride (7.5 ml) and concentrated sulfuric acid (0.5 ml) were added to the sirup and the solution was heated for 1 hour at 50-60° in a water bath. After cooling for 5 minutes, the acetylation mixture was poured slowly with stirring into about 70.0 ml of ice-water. The mixture was transferred to a separatory funnel and the alditol acetates were extracted with three successive amounts of freshly distilled methylene chloride (25.0 ml, 15,0 ml and 10.0 ml). The methylene chloride extract was concentrated to dryness on a rotary evaporator at 75°. Distilled water (1.0 ml) was added to the residue and re-evaporated. The alditol acetates were dissolved in 2.0 ml of freshly distilled methylene chloride and about 4.0 µl of the solution were injected into the gas chromatograph (21, 33). The peaks in the resulting spectra wery identified by comparison of retention times with authentic known alditol acetates and by peak enhancement techniques. The areas under the peaks in the resulting spectra were measured by means of a planimeter.

H. Characterization of the Carbohydrates Solubilized by the Acidified Sodium Chlorite Delignification Reaction

(Isolation described in section II-F)

1. Ash Determination

The inorganic ash was determined by a modification of the procedure of Paech and Tracey (34).

Six samples (0.170 g to 0.180 g dry weight) were weighed into silica dishes. The samples were saturated with concentrated sulfuric acid (0.2 ml). The mixtures were stirred thoroughly with glass rods and set aside for 1-1½ hours. They were placed in a muffle furnace and heated gently until charring occurred. The temperature was increased to 300° to drive off the sulfuric acid (about 30 min). The temperature was increased to 500° and heating continued until ashing was complete (about 5 hours). The ashed samples were placed in a dessicator to cool and were weighed after 20 min. The ash content was 13.39±1.57%.

2. Dialysis and Precipitation with Ethanol

In an attempt to separate and purify the carbohydrates in the acidified sodium chlorite soluble solids, a sample was dialyzed and re-precipitated (35).

An aliquot (22.2 g, dry weight) was dissolved in distilled water (500 ml) and dialyzed against distilled water. The water was changed each day for four days, concentrated to about 200 ml under reduced pressure on a rotary evaporator and lyophilized to yield a brown solid; weight 5.6 g or

22.4% of the starting material. An aliquot of the fraction was subjected to paper chromatography, under conditions to be described in detail later.

The solution in the dialysis bag was concentrated under reduced pressure on a rotary evaporator to a volume of 400 ml. The concentrate was added, with stirring, into 1200 ml of 95% ethanol to provide a 70-71% ethanol concentration resulting in the formation of a flocculent white precipitate. The mixture was allowed to stand at room temperature for several hours and then centrifuged. The decantate was concentrated and lyophilized to yield a fluffy white powder; weight 7.4 g or 33.3% of the starting material.

The white residue was lyophilized to yield a fluffy white powder; weight 7.7 g or 34.7% of the starting material; ash content 11.88±2.83%.

Dialysis and precipitation did not lower the ash content of the original material beyond the degree of error of the ash determinations, and so all further work was done on the original material.

3. Elemental Analysis for Nitrogen, Sulfur, Phosphorus and the Halogens

The procedures used are slight modifications of the Lassaigne's sodium fusion method (36, p. 1039). A small glass test-tube was supported in a clamp and a small cube of sodium metal was added. The tube was gently heated in a flame until the sodium melted and the vapors rose 1-2 cm up the walls of the tube. A small amount of solid was added directly onto the molten sodium. The tube was strongly heated over an open flame until the entire end was red hot. The heating was continued for one or two minutes and the tube was then plunged into an evaporating dish containing about 10 ml of distilled water so that the hot tube shattered. The mixture was heated to boiling, the insolubles removed by filtration, and the filtrate recovered for elemental analysis.

An aliquot (2-3 ml) of the filtrate was added to a test-tube containing about 0.1 g of powdered ferrous sulfate. The mixture was gently heated with shaking until it boiled. Without cooling, sufficient dilute sulfuric acid was added to dissolve the iron hydroxide and give the solution an acid reaction. A precipitate of Prussian blue formed, indicating the presence of nitrogen.

A small aliquot (15.0 mg dry weight) of the acidified sodium chlorite soluble material was quantitatively analyzed (Pascher and Pascher, 53 Bonn, Buschstrasse 54, West Germany); nitrogen content, 0.84%.

A second aliquot (2.0 ml) of the filtrate from the sodium fusion reaction was acidified with dilute acetic acid and a few drops of lead acetate was added. No reaction resulted, indicating that no sulfur was present.

A third aliquot (1.0 ml) of the filtrate from the sodium fusion reaction was acidified with 3.0 ml of concentrated nitric acid and boiled for one minute. The solution was cooled and an equal volume of ammonium molybdate reagent was added. The solution was warmed to 40-50° and allowed to stand but no yellow precipitate formed, indicating that no phosphorus was present.

A fourth aliquot (2.0 ml) of the filtrate from the sodium fusion reaction was acidified with dilute sulfuric acid and boiled gently until it had been reduced to about 1 ml to remove any hydrogen cyanide which might be present. A few drops of aqueous silver nitrate was added. No precipitate formed, indicating that no halogens were present.

4. Strong Acid Hydrolysis

An aliquot (177.6 mg dry weight) of the solids solubilized by the acidified sodium chlorite reaction was dissolved in 77% sulfuric acid (3.0 g) and allowed to stand for 30 minutes at room temperature. Water

(56.29 g) was slowly added with stirring to provide a 3.9% sulfuric acid solution. The solution was refluxed for 5 hr., cooled to room temperature, and neutralized to pH 5.0 by titration with a saturated aqueous barium hydroxide solution. The resulting precipitate of barium sulfate was removed by centrifuge and washed well with water. The decantate plus washings were concentrated on a rotary evaporatory to 50 ml.

5. Mild Acid Hydrolysis

A part (177.6 mg dry weight) of the solids solubilized by the acidified sodium chlorite reaction was dissolved in 3% sulfuric acid (50 ml) and the solution was refluxed 5 hr. After cooling to room temperature the solution was titrated to pH 5.0, with a saturated solution of aqueous barium hydroxide. The resulting precipitate of barium sulfate was removed by centrifuge and washed well with water. The decantate plus washings were concentrated to 25 ml on a rotary evaporator.

6. Qualitative Amino Acid Analysis by Paper Chromatography

The hydrolyzates from the strong acid treatment and the mild acid treatment were subjected to two dimensional paper chromatography (Whatman No. 1 paper) using water-saturated phenol (beakers of 0.3% ammonium hydroxide were placed in the bottom of the developing tank) as developer in one direction and n-butanol-formic acid-water (20:6:5 v/v/v) as developer in the second direction. After air-drying, the papers were sprayed with ninhydrin spray reagent (0.02% ninhydrin in n-butanol) and heated at 100±5° in an oven for 5 minutes (37, pp. 93-96).

7. Qualitative Carbohydrate Analysis by Paper Chromatography

The hydrolyzates from the strong acid hydrolysis and the mild acid hydrolysis were subjected to paper chromatography using ethyl acetate-pyridine-water (8:2:1 v/v/v) as developer. The solvent was allowed to migrate almost to the bottom of the papers at which time they were removed from the tank and air-dried. The papers were returned to the tank and developed as before (repeated 3 times). The paper chromatograms were sprayed with o-aminodiphenyl reagent (0.4 g o-aminodiphenyl dissolved in a solution prepared from 100 ml glacial acetic acid and 20 ml distilled water) and heated at 100±2° in an oven for 5 min (19).

8. Quantitative Carbohydrate Analysis by Gas-Liquid Chromatography

The gas-chromatograph used was a Hewlett-Packard 5751B Research Chromatograph (Hewlett-Packard Company, Palo Alto, California) equipped with dual flame ionization detectors. The conditions were: column, 6.5% ECNSS-M on Gas Chrom Q 100/120 mesh, 6 ft. x 1/8 in. O.D. stainless steel; injection port 200°; detector 230°; column temperature 180° isothermal; helium flow 30 ml/min; range setting 10^2, attenuation setting 16.

The solids solubilized by the acidified sodium chlorite reaction were hydrolyzed and the derivatives prepared for injection into the gas chromatograph as follows. A portion of the solids (0.2721 g, dry weight) was dissolved in 3% sulfuric acid (80 ml) and refluxed for 5 hours. After cooling, authentic myo-inositol (0.1004 g) was added. The solution was neutralized to pH 5.0 with a saturated aqueous solution of barium hydroxide. The resultant precipitate of barium sulfate was removed by centrifuge and the decantate plus washings were concentrated to about 25 ml and transferred to a 100-ml round-bottomed flask. Sodium borohydride (0.08 g) was added to the flask and allowed to react for 2 hr. at room temperature (30).

The excess sodium borohydride was decomposed by adding acetic acid until gas evolution ceased. The solution was concentrated to a sirup on a rotary evaporator and methanol (10 ml) was added and re-evaporated. The addition and removal of methanol was repeated five time (32). The resulting sirup was dried in an oven at 105° for 15 min to ensure complete removal of water.

Acetic anhydride (7.5 ml) and concentrated sulfuric acid (0.5 ml) were added to the sirup and the solution was heated for 1 hour at 50-60° in a water bath. After cooling for 5 min. the acetylation mixture was poured slowly with stirring into about 70 ml of ice water. The mixture was transferred to a separatory funnel and the alditol acetates were extracted with three successive amounts of methylene chloride (25 ml, 15 ml, and 10 ml). The methylene chloride extract was concentrated to dryness on a rotary evaporator at 75°. Water (1 ml) was added to the residue and re-evaporated. The alditol acetates were dissolved in 2 ml of methylene chloride and about 1.0 μl of the solution was injected into the gas chromatograph for quantitative analysis (31).

The areas under the peaks in the resulting spectrum were measured with a planimeter.

9. Qualitative Uronic Acid Analysis by Infrared Spectroscopy

A sample (0.5 mg) of dried solids solubilized by the acidified sodium chlorite with potassium bromide (200 mg) and pressed into a pellet. A spectrum was taken of the pellet over the range of 4000 to 300 cm^{-1} (Beckman IR-20A). The residue from the 0.5% ammonium oxalate extraction was also analyzed by infrared spectroscopy.

An infrared spectrum was also obtained in a Nujol mull prepared by grinding it to a thick paste in Nujol oil (paraffin oil).

10. Qualitative Uronic Acid Analysis by Color Reactions

Concentrated sulfuric acid (6.0 ml) was added slowly to an aqueous solution (1.0 ml) of the 3% sulfuric acid hydrolyzate of the solids solubilized by the acidified sodium chlorite reaction and cooled under tap water. The reaction mixture was heated for 20 min in a boiling water bath and cooled. An aliquot (0.2 ml) of a 0.1% ethanolic solution of carbazole was added and the test sample allowed to stand for 2 hours at room temperature. The development of a purple color indicated a uronic acid. The solution was scanned in the ultraviolet range and showed maximum absorption at 535 nm indicating the presence of a uronic acid (38, p. 497).

An aliquot (177.6 g dry weight) of the solubilized solids was dissolved in 3% sulfuric acid (50 ml) and the solution was refluxed for 5 hr. The hydrolyzate was neutralized by passage through a column of Amerlite IRA-400 anion exchange resin in the -OH form. An amount (1.0 ml) of the neutralized solution was tested for uronic acids by the carbazole-sulfuric acid test described above. The results were positive.

A small aliquot of the 0.5% ammonium oxalate residue was also tested with carbazole-sulfuric acid. The results showed a strong positive test. Color tests were also run on authentic galacturonic acid (positive test), oxalic acid and benzoic acid (negative tests).

11. Qualitative Uronic Acid Analysis by Paper Chromatography

The hydrolyzate prepared from the mild acid hydrolysis of the solids solubilized by the acidified sodium chlorite reaction was subjected to paper chromatography. Whatman No. 1 filter paper was employed in conjunction with the following solvent systems:

1. water saturated n-butanol-absolute ethanol-water (10:9:1 v/v/v)
2. water saturated n-butanol-acetone-water (4:5:1 v/v/v)
3. ethyl acetate-pyridine-water (8:2:1 v/v/v).

Authentic galacturonic acid was chromatographed simultaneously with the unknown hydrolyzate.

For the first two solvent systems, the papers were first impregnated with a phosphate buffer solution prepared by titrating a 0.01 M solution of disodium hydrogen phosphate to pH 5.0 with a 0.1 M solution of phosphoric acid (39).

The chromatograms developed with solvent three were alternately irrigated and air-dried for a total of 6 times. They were thus in the solvent atmosphere for a total of about 30 hr.

After development, the paper chromatograms prepared from the first two solvent systems were sprayed with aniline hydrogen phthalate reagent (1.66 g of phthalic acid dissolved in 100 ml of water-saturated n-butanol containing 0.93 g of freshly distilled aniline) (40). The chromatograms prepared from the third solvent system were sprayed with the o-aminodiphenyl reagent. After spraying, all chromatograms were heated in an oven at 100±2° for 5 min.

12. Preparation of Methyl Ethers

An aliquot of the solids solubilized by the acidified sodium chlorite reaction (1.461 g dry weight) was dissolved in 18% aqueous sodium hydroxide at 0° with stirring followed by the addition of 6.0 g of sodium hydroxide pellets. Sodium hydroxide (100 ml, 30%) and dimethyl sulfate (50 ml) were added simultaneously over a period of 3 hours while maintaining the temperature at 0°. Acetone (150 ml) was added to prevent foaming and the mixture was stirred for an additional 45 hr at room temperature. The solution was cooled to 0°, neutralized with 10% sulfuric acid, dialyzed against water (3 days), concentrated under reduced pressure (200 ml), lyophilized, and the entire methylation sequence repeated (41).

The product was further methylated by the method of Falconer and Adams (42) by solution in 150 ml of tetrahydrofuran followed by treatment over a period of 60 hours with seven 10 g portions of crushed sodium hydroxide, each followed by a 12 ml portion of dimethyl sulfate. The solution was stirred vigorously and additional solvent was added as needed to maintain fluidity. The product was recovered as described above for the first methylation, and the concentrated dialyzate (600 ml) was extracted with three 300 ml portions of chloroform. The chloroform layer was concentrated under reduced pressure, yielding a light brown solid which was dissolved in acetone and filtered. The concentrated filtrate (50 ml) was poured into petroleum ether (b.p. 30-60°, 200 ml) and the white precipitate which formed was recovered by centrifuge (after the mixture was refrigerated for 6 days) and lyophilized, affording a white, fluffy powder; yield 80 mg.

13. Acid Hydrolysis of the Methyl Ethers

An aliquot (40 mg) of the methylated material prepared above was hydrolyzed for 3 hr at 97° with 88% formic acid (10 ml). The formic acid was removed by evaporation under reduced pressure followed by the addition and removal of water, then hydrolyzed with 0.5 N sulfuric acid (5 ml for 2.5 hr at 97°). Upon cooling the solution was neutralized with water-saturated barium hydroxide and the solids were removed by centrifugation. The decantate was concentrated to about 2 ml of sirup. The sirup was chromatographed on paper using the developer ethyl acetate-pyridine-water (8:2:1 v/v/v).

I. Characterization of the Holocellulose Fraction

1. Elemental Analysis for Nitrogen, Sulfur, Phosphorus, and the Halogens

To a small test tube (75 x 12 mm), supported in a clamp, was added 0.06 g of sodium metal. The tube was heated in a Bunson Burner flame until the sodium melted and the vapors rose 1-2 cm up the walls of the tube. The tube was strongly heated over an open flame until the entire end was red hot.

The tube was then plunged into a small beaker containing 10 ml of distilled water. The hot tube was shattered. The mixture was heated to boiling. The insolubles were removed by filtration, and the filtrate recovered for elemental analysis.

A second 10 ml of filtrate from the sodium fusion reaction was similarly prepared.

An aliquot (2.5 ml) of the above filtrate was added to a test tube containing about 0.1 g of powdered ferrous sulfate. The mixture was gently heated with shaking until it boiled. Without cooling, just enough dilute sulfuric acid was added to dissolve the iron hydroxide and give the solution an acid reaction. No precipitate of Prussion Blue formed, indicating the absence of nitrogen.

A known nitrogen containing compound, alanine (0.02 g), was fused with sodium as described above. A precipitate of Prussian Blue formed upon reacting the acidified sodium fusion solution with ferrous sulfate indicating the presence of nitrogen.

A second aliquot (2.0 ml) of the filtrate from the sodium fusion reaction was acidified with dilute acetic acid. A few drops of lead acetate were added. No yellow precipitate formed which indicated no sulfur.

A known sulfur-containing compound, cystine, was fused with sodium as above. A yellow precipitate formed in the filtrate by acidifying with acetic acid and adding a few drops of lead acetate. This indicated the presence of sulfur.

A third aliquot (2.0 ml) of the filtrate from the sodium fusion reaction was acidified with 3.0 ml of concentrated nitric acid and boiled for one minute. The solution was cooled and to it was added an equal volume of ammonium molybdate reagent. The solution was warmed to 40-50° and allowed to stand. No yellow precipitate formed which indicated the absence of phosphorous.

A known phosphorus containing compound, glucose-1-Phosphate was fused with sodium as described above. A yellow precipitate formed upon reacting the acidified sodium fusion solution with ammonium molybdate indicating the presence of phosphorus.

A fourth aliquot (2.0 ml) of the filtrate from the sodium fusion reaction was acidified with dilute nitric acid and added to an excess of silver nitrate solution. No precipitate formed indicating the absence of halogens.

Known sodium chloride (0.02 g) was fused with sodium as described above. A precipitate formed when the acidified filtrate was added to a silver nitrate solution, indicating the presence of a halogen. The mother liquor was decanted and the precipitate was treated with dilute aqueous ammonia solution. A white precipitate formed which was readily soluble in the ammonia solution indicating the presence of chlorine.

2. Determination of Lignin

a. Acid-Insoluble Lignin

A portion (0.4566 g, dry weight) of bark holocellulose was placed in a 500-ml, three-necked round-bottomed flask submerged in a cold-water bath (18-20°). An amount (10.0 ml) of cold (13-15°) 72% sulfuric acid was added slowly with stirring. The sample was allowed to stand, with frequent stirring, for 2 hours at 18-20°. The holocellulose became completely dispersed in the acid. The transparent sirup was diluted to 3.0% sulfuric acid concentration by slowly adding 376.0 ml of distilled water. The sample was refluxed for 4 hours. The insoluble material was recovered by filtration using a Gooch crucible (fine porosity) which had previously been dried and weighed. The residue in the crucible was washed free of acid with 150.0 ml of hot water and dried in an oven at 105±2° until the weight became constant (18 hours) (43). The average of three determinations was 0.0139 g or 3.05% of the holocellulose.

b. Acid-Soluble Lignin

The filtrates from the above Klason lignin determinations were combined to yield a solution of 1544.0 ml. The solution was analyzed for the acid-soluble lignin content by the characteristic lignin absorptions at 280 nm and 210 nm (44, 45, p. 259). The instrument used was a Beckman ACTA TM III UV-Visible spectrophotometer. The instrument was standardized by placing 3.0 ml of 3.0% sulfuric acid solution in both the reference and sample cells (cell widths, 1.0 cm) and scanning over the ultraviolet spectral range from 370 nm to 200 nm. The sample cell was cleaned, dried and 3.0 ml of the acid filtrate from the Klason lignin determination was added. The sample was scanned over the ultraviolet spectral range from 320 nm to 200 nm. Absorption peaks were recorded at 280 nm (absorbance 2.60) and at 210 nm (absorbance 3.77).

3. Hydrolysis with 77.0% Sulfuric Acid

Holocellulose (913.2 mg, dry weight) was dissolved in 12.4 g of 77.0% sulfuric acid in a 1-liter three-necked, round-bottomed flask, at ice-water temperature for 1 hour. The transparent sirup of dissolved holocellulose was diluted to 3% sulfuric acid by the dropwise addition of 247.0 ml of distilled water from a dropping funnel. Small, insoluble particles were observed in the dilute solution. The dilute solution was refluxed for six hours. The small, insoluble particles remained. The solution was filtered. The filtrate was neutralized to pH 5 with saturated aqueous barium hydroxide solution. The resulting precipitate of barium sulfate was removed by centrifuge and washed well with water. The decantate plus washings were concentrated on a rotary evaporator to a sirup.

4. Qualitative Carbohydrate Analysis by Paper Chromatography

The hydrolyzate from the sulfuric acid hydrolysis was separated into its component monosaccharides by paper chromatography. Three drops of the hydrolyzate sirup was dissolved in 1.0 ml of distilled water. Five micropipettes of the above dilute hydrolyzate were spotted on Whatman No. 1 chromatographic paper. A solution of a known monosaccharide mixture containing 1.0% each of glucose, galactose, mannose, arabinose and xylose were placed along with the unknown hydrolyzate on the front of the chromatographic paper 3 cm from one end. The chromatographic sheet was developed by the descending method in a chromatographic tank of dimensions 61 cm x 67 cm x 82 cm in height, pre-saturated for one day with the developer ethyl acetate-pyridine-water (8:2:1 v/v/v). The solvent was allowed to migrate almost to the bottom of the papers (6-8 hr). The papers were removed from the tank and air dried. They were placed back in the tank and developed again as above (repeated 3 times). The papers were sprayed with o-aminodiphenyl reagent (0.4 g o-aminodiphenyl dissolved in a solution prepared from 100.0 ml of glacial acetic acid and 20.0 ml of distilled water) and heated at 100±2° in an oven for 5 min to develop the color. The monosaccharides after separation and reaction with the color reagent were investigated under ultraviolet light. Hexoses showed a white color and pentoses showed a red brick color under the ultraviolet light.

5. Isolation of Crystalline Sugar Derivatives

a. Isolation of Sugars by Preparative Paper Chromatography

A part (6.0 g, dry weight) of bark holocellulose was added to 77.0% sulfuric acid (90.0 g) in a 5-liter round-bottomed flask which was submerged in an ice-water bath. The mixture was stirred for 1 hr at the end of which time it appeared as a transparent sirup. Water (1688.0 ml) was added slowly to the sirup to dilute the sulfuric acid to 3% concentration. The solution was refluxed for a total of six hours. It was allowed to cool to room temperature and neutralized to pH 5.0 by the careful addition of aqueous saturated barium hydroxide solution. The resulting precipitate of barium sulfate was removed by centrifugation and washed well with water. The decantate plus washings were concentrated to about 20.0 ml on a rotary evaporator.

Aliquots of approximately 0.1 ml each of the concentrated hydrolyzate were streaked with a syringe across large pieces of Whatman No. 3 MM chromatographic paper, 9 cm from one end of the paper. After the streaks were dry, the papers were developed by the descending method using the solvent system ethyl acetate-pyridine-water (8:2:1 v/v/v). The solvent was allowed to migrate almost to the bottom of the papers (about 6-8 hr). The sheets were removed and dried in the air overnight. The sheets were developed and air-dried again (repeated 5 more times). A strip 1.0 cm in width was cut from the center of each sheet along the direction of solvent migration. The strips were sprayed with o-aminodiphenyl reagent and dried in an oven at 105±3° for 10 min. The degree of separation was examined under ultraviolet light. It required six solvent migrations to the bottom of the sheets to adequately separate the monosaccharides (glucose, galactose, mannose, arabinose, and xylose) in the holocellulose hydrolyzate.

After assurance that the sugars were well separated, 1.0 cm strips were cut from the papers in the direction of solvent flow at 6 cm intervals across the width of the papers. These strips were sprayed with o-aminodiphenyl indicator as before. After location of the sugars, the strips were re-fitted back into the original paper chromatograms and the unsprayed cross-bands of each sugar cut out. The sugars were collected from 24 such paper chromatograms.

The paper bands thus collected were placed in 1 liter beakers (a different beaker for each sugar), and the sugars eluted with 800.0 ml of distilled water. The eluate of each isolated sugar was concentrated on a rotary evaporator to a sirup. Methanol was added to the sirup and then re-evaporated (repeated 3 times), and final drying was achieved in a dessicator.

Each isolated sugar sirup was re-chromatographed on paper simultaneously with a solution of known sugars (glucose, galactose, mannose, arabinose and xylose; each 1% concentration). In this way each isolated sugar sirup was of assured purity before preparation of a crystalline derivative was attempted.

b. Diethyl Dithioacetal Acetate Derivatives of Galactose and Arabinose

Diethyl dithioacetal acetate derivatives of known monosaccharides were synthesized for direct comparison with derivatives of the sugars from the holocellulose hydrolyzate. The general procedure for each known sugar was that reported by Wolfrom and Karabinos (46). Galactose is used as a typical reaction illustration. Galactose (100.0 mg) was dissolved in concentrated hydrochloric acid (1.0 ml, 12 N) in a 100-ml round-bottomed flask submerged in an ice-water bath. Ethyl mercaptan (1.0 ml) was added under a well ventilated hood and the mixture was stirred for one hour. The mixture was neutralized at ice-bath temperature by the addition of ammonium hydroxide (13-14 drops, 15 N). During neutralization, white smoke resulted and a green precipitate was formed. The mixture was concentrated to dryness under aspirator vacuum on a rotary evaporatory at 40°. Dryness was accomplished by the addition and re-evaporation of absolute ethanol (repeated 5 times). The greenish-yellow color disappeared after the second or third evaporation of ethanol, and a white solid resulted.

The white solid was acetylated with a mixture (6.0 ml) of acetic anhydride and anhydrous pyridine (2:1 v/v). The reaction was allowed to continue overnight at room temperature. The solution was poured into distilled water (10.0 ml) and extracted twice with chloroform (15.0 ml each time). The chloroform extract was washed three times with saturated aqueous sodium bicarbonate (10.0 ml each time). The acid-free chloroform extract was concentrated on a rotary evaporator to a sirup. Crystallization was attempted by dissolution of the sirup in methanol followed by the dropwise addition of water until a permanent cloudiness resulted. Crystallization of some of the derivatives was also attempted from 95% ethanol.

Diethyl dithioacetal acetate derivatives of known mannose, galactose, arabinose and xylose were similarly prepared. However, only galactose diethyl dithioacetal acetate (m.p. 75.0-77.0°; literature value 76.5-77.0°), and arabinose diethyl dithioacetal acetate (m.p. 78.0-79.0°; literature value 79.0-80.0°) crystallized. Therefore, similar derivatives of only the galactose and arabinose sirups isolated from the holocellulose were prepared: galactose diethyl dithioacetal acetate, m.p. 76.5-77.5°, unchanged on admixture with authentic material $[\alpha]_D^{23.5}$ + 10.0° (c 4.0, chloroform) arabinose diethyl dithioacetal, m.p. 77.0-78.5°, unchanged on admixture with authentic material $[\alpha]_D^{23.5}$ - 26.40 (c 1.54, chloroform) literature value -30.0°.

c. Acetate Derivitave of Glucose

An amount (5.4 ml) of acetic anhydride was heated to about 100° and 0.44 g of anhydrous sodium acetate was added. In small portions, 1.0 g of D-glucose was added with stirring over the course of 30 min.

When the addition was completed, the solution was cooled to room temperature, poured into 16 ml of ice-water, and stirred for 30 min. The acetylated product was extracted with three 20-ml portions of chloroform. The extracts were washed with 20 ml of distilled water and concentrated on a rotary evaporator to a sirup. Glucose pentacetate was crystallized by dissolution of the sirup in methenol followed by the dropwise addition of water until a permanent cloudiness resulted. After two recrystallizations from 95% ethanol, white crystalls of glucose pentaacetate were obtained; (yield 234.0 mg, m.p. 133.5-135.0; literatuve value 135°) (47).

Glucose sirup (250 mg) isolated from the bark holocellulose hydrolyzate by preparative paper chromatography was similarily acetylated. White crystals of glucose pentaacetate were obtained; yield 112 mg, m.p. 133-134°, unchanged on admixture with authentic material, $[\alpha]_D^{23.5}$ + 3.9° (c 3.4, chloroform), literature value 3.8°.

d. Di-O-benzylidene Dimethyl Acetal Derivative of D-Xylose

The reagent for preparing di-O-benzylidene dimethyl acetal derivatives of D-xylose was prepared by dissolving 2 ml of redistilled benzaldehyde in a mixture of 2.5 N methanolic hydrogen chloride (1.0 ml) and spectroquality methanol (6.0 ml). An aliquot (2.5 mg) of D-xylose was treated with 5.0 ml of the reagent at room temperature. After standing for 1 hr, silky needles appeared in the solution. After standing for 6 hr the solution solidified. The white crystals were recovered by filtration, washed with 200 ml of ice-water and dried in a dessicator for 3 days. When recrystallized from spectroquality methanol, silky needles of di-O-benzylidene-D-xylose dimethyl acetal were obtained; m.p.210-211.5°, literature value 211-212° (48, p. 88).

Xylose sirup isolated by preparative paper chromatography from bark holocellulose hydrolyzate was similarily treated with the prepared reagent and crystalline needles were obtained. After recrystallization from spectroquality methanol, silky needles of di-O-benzylidene-D-xylose dimethyl acetal were obtained; yield 90.7 mg, m.p. 211-212.5°, unchanged on admixture with authentic material, $[\alpha]_D^{23.5}$ - 8.0° (c 1.0, chloroform) literature value -7.0° (48, p. 88).

e. Phenylhydrazone Derivative of Mannose

D-mannose (1.0 g) was added to a solution of phenylhydrazine (1.0 ml) in 95% ethanol (15.0 ml) in a 50-ml round-bottomed flask. The mixture was warmed for 30 min in a hot-water bath (60°) and then placed in the refrigerator overnight. The resulting crystals of mannose phenylhydrazone were removed by filtration and washed successively with a few drops each of water, ethanol, and diethyl either; yield 94.5 mg, m.p. 191-192°. The crystals were again washed with a few drops each of water, ethanol and diethyl ether; yield 90.5 mg of glistening snow-white crystals, m.p. 194-195°, $[\alpha]_D^{23.5}$ + 2.5° (c 0.1 pyridine), literature value 199.0-200.0° $[\alpha]_D^{26}$(c 0.1 pyridine) (49, p. 147).

An aliquot (9.0 g air-dried weight, moisture content 8.68%) of holocellulose was hydrolyzed with 77.0% sulfuric acid as previously described. One-third of the resulting hydrolyzate was concentrated to a sirup. The sirup was dissolved in 95% ethanol (45.0 ml) followed by the addition of phenylhydrazene (3.0 ml) (49, p. 107). The mixture was warmed for 30 min in a hot-water bath (55°), cooled, and kept in the refrigerator overnight. The resulting white crystals were recovered by filtration and washed successively with water, ethanol and diethyl ether; m.p. 160-180°. These crystals were dissolved in water (50.0 ml) and kept in the refrigerator overnight. The insoluble white crystals were recovered by filtration and dried; yield 226.7 mg, m.p. 193.5-194.5, unchanged on admixture with authentic mannose phenylhydrazone; $[\alpha]_D^{23.5}$ + 25° (c 0.1, pyridine) literature value +26° (49, p. 147).

Another aliquot (5.0 g air-dry weight, moisture content 8.68%) of holocellulose was refluxed with 90% formic acid (50.0 ml) for 1 hr (41). The residue was removed by centrifugation and filtration. The filtrate was concentrated to a sirup which was hydrolyzed with 0.5 N sulfuric acid (50.0 ml) under reflux for 2.5 hr. The acid hydrolyzate was neutralized to pH 5.0 with a saturated aqueous solution of barium hydroxide and the resulting precipitate was removed by centrifugation. The remaining inorganic salts were removed by refluxing the concentrated sirup with absolute methanol followed by filtration (repeated four times) and removal of solvent.

The resulting sirup was dissolved in 95% ethanol (15.0 ml) followed by the addition of phenylhydrazine (1.0 ml) (49, p. 147). The solution was warmed (55°) for 30 min and then kept in a refrigerator overnight. The resulting white crystals were recovered by filtration washed successively with water, ethanol, diethyl ether and dried; yield 132.4 mg; m.p. 194-196°, unchanged on admixture with authentic mannose phenylhydrazone; $[\alpha]_D^{23.5}$ + 25° (c 0.1, pyridine), literature value +26° (49, p. 147).

6. Quantitative Determination of Reducing Sugars by Copper Reduction

a. Standardization of Sodium Thiosulfate Solution

Potassium iodate (1.41387 g) was dissolved in distilled water and the solution was diluted to 1 liter in a volumetric flask. An aliquot (100.0 ml) of the solution was removed with a pipette. A 1.8 M potassium iodide solution (10.0 ml) and a 12 M hydrochloric acid solution (3.0 ml) were added to the aliquot. This solution was titrated with the sodium thiosulfate solution to be standardized (about 0.005 M) to a starch end-point. The exact molarity (0.0058 M) of the sodium thiosulfate solution was calculated from the average of triplicate samples (50, p. 460; 51, p. 379).

b. Preparation of Somogyi Copper Reagent

Rochelle salt (40.0 g) (potassium sodium tartrate), disodium hydrogen phosphate heptahydrate (53.0 g), and 1.0 N sodium hydroxide (100.0 ml) were dissolved in 500.0 ml of water and 80.0 ml of an aqueous solution containing 8.0 g of cupric sulfate pentahydrate was stirred in. Finally, 180.0 g of anhydrous sodium sulfate was added and dissolved. The solution was diluted to 1 liter and allowed to stand for 3 days (52, p. 383-386). Potassium iodate (37.2 mg) was added to a 100-ml aliquot of the reagent to provide a concentration of 3.72 mg per liter

c. Determination of Total Reducing Sugars in the Holocellulose Hydrolyzate

Holocellulose (300 mg air-dry weight; moisture content 8.68%) was dissolved in 77% sulfuric acid (4.50 g) in a 150-ml three-necked round-bottomed flask in an ice-bath. The mixture was diluted to 3.9% sulfuric acid concentration by the dropwise addition of water. The solution was heated to reflux and aliquots (2.0 ml) were withdrawn at 30 min intervals.

Each aliquot was neutralized to pH 2.0 with 3% aqueous sodium hydroxide solution (1.28 ml). Each neutralized solution was diluted to 25.0 ml in a volumetric flask. Three aliquots (5.0 ml each) of these solutions were added with a pipette to test tubes (20 cm x 2.4 cm). Similarly, 5.0 ml of three standard glucose solutions containing 1.0 mg, 0.75 mg and 0.5 mg per 5 ml of solution were added to identical test tubes. A blank of 5.0 ml of water was added to another test tube. Somogyi reagent (5.0 ml) was pipetted into each of these test tubes under a stream of nitrogen gas. The solutions were heated in a boiling water bath for 10 min and cooled in a water bath for 10 min. Each solution was oxidized by adding 2.5% aqueous potassium iodide (0.5 ml) and 2 N sulfuric acid (1.5 ml) and each was allowed to stand for 5 min with occasional shaking. The solutions were titrated to a starch end point with standarized sodium thiosulfate (0.0058 M) within 30 min. The amount of copper reagent reduced by the reducing sugars was obtained by subtraction of the titer of the sample from the titer of the blank. The amount of reducing sugar in the test tubes of the unknown hydrolyzate was calculated by direct comparison with the known amount of glucose in the standard solution (22).

The initial sample of holocellulose was refluxed a total of 10 hr and then heating was discontinued while the aliquots which had been withdrawn were analyzed. The titration results showed that monosaccharides were still being released after 10 hr of reflux. Therefore, a second sample of holocellulose (300 mg) was hydrolyzed by sulfuric acid as before but the reflux time was extended to 20 hr. Aliquots (2.0 ml) were removed every two hours and analyzed for reducing sugars with the Somogyi reagent. The reducing power of the solution ceased to increase after 12 hr of reflux, indicating that hydrolysis was complete at this time.

7. Quantitative Carbohydrate Analysis by Gas-Liquid Chromatography

a. Preparation of Authentic Alditol Acetates

Galactitol (0.50 g) was added to 10.0 ml of an acetylating reagent composed of pyridine-acetic anhydride (1:1 v/v). The mixture was refluxed for 1 hr and poured into ice-water (40.0 ml). A white precipitate formed which was recovered on filter paper and washed well with water. The white solid (galactitol hexaacetate) was recrystallized 3 times from 95% ethanol; m.p. 171°, literature value 171° (30, 53).

Glucitol (sorbitol), mannitol, arabinitol, and *myo*-inositol were similarily acetylated: glucitol hexaacetate, m.p. 99.5°, literature value 99-99.5° (30, 53); mannitol hexaacetate, m.p. 125.5°, literature value 126° (30, 53); arabinitol pentaacetate, m.p. 76°, literature value 76° (30, 53);

inositol hexaacetate, m.p. 215-216°, literature value 214-215° (54, p. 85).

Xylitol was acetylated as above but the derivative would not crystallize from 95% ethanol. The oil of xylitol pentaacetate was poured into ice water and extracted 3 times with chloroform. The chloroform extract was condensed to a sirup and dissolved in 95% ethanol, 85% methanol, acetone, diethyl ether, or a mixture of acetone, diethyl ether and ligroin. The sirup was dried by the addition and removal of methanol under vacuum (repeated 3 times). The xylitol pentaacetate then crystallized from acetone and was recrystallized two times, m.p. 57°, literature value 61-62° (30, 53).

Rhamnose (0.1 g) was dissolved in 100 ml of water in a 100-ml round-bottomed flask and was reduced with sodium borohydride (0.8 g) for 2 hr at room temperature. Acetic acid was added to the solution until no gas evolved. The solution was concentrated to a sirup and dried by the addition and removal of methanol on a rotary evaporator (repeated 5 times). The sirup was dried in an oven at 105° for 15 min. The dry sirup was acetylated with an acetylating reagent of acetic anhydride (75 ml) and concentrated sulfuric acid (5 ml) for 1 hr at 50-60°. The acetylated mixture was cooled for 5 min and poured into ice water and extracted with chloroform (300 ml, 300 ml, 200 ml). The chloroform extract was condensed to a sirup and dissolved in 95% ethanol. No crystals formed. The sirup was dried several times by the addition and removal of methanol on a rotary evaporator. It still did not crystallize from any solvent tried.

Each of the above crystalline alditol acetates was dissolved in turn in methylene chloride (2.0% solution) and injected (2 µl) separately into the gas chromatograph (Hewlett-Packard 5751B Research Chromatograph equipped with dual flame ionization detectors, Hewlett-Packard Company, Palo Alto, California). The conditions were: column, 6.5% ECNSS-M on Gas Chrom Q

100/120 mesh, 6 ft x 1/8 in O.D. stainless steel; injection port 180°; detector 240°; column temp 180° isothermal; helium flow 30 ml/min; range setting 10^2, attenuation setting 16.

In this way the retention time of each alditol acetate was determined. A mixture of the alditol acetates were dissolved in methylene chloride (2.0% concentration of each) and an aliquot (2 μl) of the solution was injected into the gas chromatograph. Resolution of the six compounds was excellent, and under the chromatographic conditions outlined above the retention time of each compound in the mixture was: xylitol pentaacetate 16 min; arabinitol pentaacetate 12 min; mannitol hexaacetate 28 min; galactitol hexaacetate 33 min; glucitol hexaacetate 38 min; myo-inositol hexaacetate 48 min. These retention times were used to identify alditol acetates prepared from the holocellulose hydrolyzate.

b. Determine of Optimum Gas-Chromatographic Conditions

Glucitol hexaacetate (4.63 mg), galactitol hexaacetate (0.25 mg), mannitol hexaacetate (0.75 mg), arabinitol pentaacetate (0.25 mg), xylitol pentaacetate (0.50 mg), and myo-inositol hexaacetate (6.25 mg) were dissolved together in methylene chloride (1.0 ml). Aliquots (2 μl) of the solution were injected into the gas-chromatograph under various ranges of injection port temperatures (180-230°), detector temperatures (210-270°), and column temperatures (180-185°). The areas under the peaks of the resulting spectra were measured with a planimeter. The percent recovery was calculated by comparing the peak areas of the other sugars to myo-inositol hexaacetate as 100%. The best recoveries were realized under the following conditions: injection port temperature, 180°; detector temperature, 240° and column temperature, 180°. These were the conditions generally used in this work.

c. Determination of an "Instrument K Factor"

The response of the gas chromatograph to the alditol acetates was determined by preparing 5 mixtures of the known alditol acetates in various ratio concentrations as shown in Table 5.

Each of the mixtures in Table 5 was dissolved in methylene chloride (1.0 ml) and an aliquot (2 μl) was injected into the gas chromatograph. The areas under the peaks of the resulting spectra were measured by a planimeter. The percent recovery was calculated on the basis of *myo*-inositol hexaacetate as 100%. The "Instrument K Factor" for each alditol acetate was obtained as the slope of the line obtained by plotting the ratio of the area of each alditol acetate to that of the standard (*myo*-inositol hexaacetate) against the ratio of the weight of the alditol acetate to that of the standard (*myo*-inositol hexaacetate). The resulting "Instrument K Factors" were as follows: xylitol pentaacetate 0.95; arabinitol pentaacetate 0.96; mannitol hexaacetate 0.96; galactitol hexaacetate 0.98; glucitol hexaacetate 0.92.

d. Analysis of the Holocellulose Hydrolyzate

An amount (300 mg, air-dry weight, moisture content 8.68%) of holocellulose was dissolved in 4.5 g of 77.0% sulfuric acid in an ice-bath for one hour with occasional stirring. After one hour, the translucent sirup was diluted to 3.9% sulfuric acid by dropwise addition of 84.42 ml of distilled water from a dropping funnel. The dilute solution was refluxed for 6 hr. The solution was diluted to 100 ml from which 25 ml of hydrolyzate was removed. To this 25.0 ml of hydrolyzate, 25 mg of *myo*-inositol was added. The hydrolyzate was neutralized to pH 5.0 with saturated aqueous barium hydroxide solution, centrifuged, concentrated to 25 ml and transferred to a 100-ml round-bottomed flask. Sodium borohydride (0.08 g) was added to the flask and allowed to react for 2 hr at room temperature (30).

The excess sodium borohydride was decomposed by adding acetic acid until gas evolution ceased. The solution was concentrated to a sirup on a rotary evaporator, and methanol (10.0 mg) was added and re-evaporated. The addition and removal of methanol was repeated eight times (32). The resulting solid was dried in an oven at 105° for 15 min to ensure complete removal of water.

An aliquot (8.0 ml) of a mixture containing acetic anhydride (7.5 ml) and concentrated sulfuric acid (0.5 ml) were added to the dry solid and the mixture was heated for 1 hour at 50-60° in a water bath. After cooling for 5 min the acetylation mixture was poured with stirring into about 70 ml of ice-water in a 150-ml beaker. Two immiscible layers (water layer on top and acetate layer on the bottom) formed. These were transferred to a 150-ml separatory funnel and the alditol acetates were extracted with three successive amounts of methylene chloride (25 ml, 15 ml, and 10 ml). The methylene chloride extract was concentrated to dryness on a rotary evaporator at 75°. Water (1.0 ml) was added to the sirup to remove remaining acetic anhydride. The resulting alditol acetates were dried in a dessicator. The alditol acetates (20.0 mg) were dissolved in methylene chloride and diluted to 1.0 ml in a volumetric flask (1.5%). An aliquot (2.0 µl) of the solution was injected into the gas chromatograph for quantitative analysis (31).

The areas under the peaks were measured with a planimeter and compared to the area under the peak of myo-inositol hexaacetate which represented the area from 25 mg of inositol. The weights of the other sugars were thus calculated as: glucose, 156.8 mg; galactose, 5.9 mg; mannose, 26.2 mg; arabinose, 4.6 mg; xylose, 16.5 mg; total 198.6 mg.

A second sample of holocellulose (300 mg) was hydrolyzed as above except that it was refluxed for 12 hr instead of 6 hr in 3.9% sulfuric acid. The alditol acetates were prepared and injected into the gas chromatograph as above.

The areas under the peaks were measured with a planimeter and compared to the area under the peak of myo-inositol which represented the area from 25 mg of sugar. The weights of the other sugars were thus calculated as: glucose, 160.4 mg; galactose, 5.8 mg; mannose, 27.9 mg; arabinose, 7.8 mg; xylose, 21.8 mg; total 223.6 mg. This increase in sugar return after 12 hr of reflux over the 6 hr of reflux is in agreement with the Somogyi values for total reducing sugars which had indicated that a minimum reflux time of 12 hr was required to completely hydrolyze the holocellulose.

J. Fractionation of the Holocellulose into its Component Polysaccharides

1. Impregnation with 2.0% Barium Hydroxide, then Extraction with 10.0% Aqueous Potassium Hydroxide Solution

Air-dried holocellulose (54.3 g, moisture content 8.68%) from Douglas-fir inner bark was slurried for 20 min at 25° in 779.0 g of aqueous barium hydroxide containing 63.4 g of barium hydroxide octahydrate. The consistency of the slurry was 6.0% based on the holocellulose content and the concentration of barium hydroxide in the liquor was 4.4%. At the end of the 20 min period 952.0 g of 18.38% aqueous potassium hydroxide solution was added which lowered the slurry consistency to 2.81% and the barium hydroxide concentration to 2%. The potassium hydroxide concentration of the extraction liquor was 10.0%. Treatment was continued for a second 20 minute period at 25°. Separation was attempted by filtration through Whatman No. 1 filter paper and a filter crucible. It was difficult to filter the colloidal solution. The difficulty was overcome by filtration through milk filter-disks (rapid). The solids were washed with distilled water, dialyzed for three days, freeze-dried and weighed; weight 42.3 g or 84.6% of the starting holocellulose. They were termed "Residue A" (55).

The filtrate plus the washings were acidified with acetic acid to a pH of 5.0 and condensed to 500.0 ml on a rotary evaporator. The polysaccharide solids were precipitated from the filtrate by the addition of 500.0 ml of methanol. The precipitate was recovered by centrifuge and redispersed in 700 ml of 70% methanol. The re-precipitation was repeated 4 times. Methanol was removed from the precipitate by the addition of water followed by evaporation using a ratuvapor. The solids were recovered by freeze-drying; weight 3.5 g or 7.0% of the original holocellulose. They were labeled "Hemicellulose A" (55).

2. Extraction with 1.0% Aqueous Sodium Hydroxide Solution

Residue A (41.4 g dry weight) was extracted with a 1% aqueous sodium hydroxide solution by adding it to 990.0 g of a 9.97% aqueous sodium hydroxide solution to give a 4.0% slurry consistency based on the content of Residue A. The mixture was stirred for 20 min at 25°. The solids were recovered by filtration using milk filter disks. The residue was washed with 250 ml of 1% sodium hydroxide solution followed by 1 liter of distilled water. The solids were transferred with water to a dialysis bag and the mixture was dialyzed for 3 days against running water. The solids which remained in the dialysis bag were recovered by freeze-drying and weighed; weight 39.1 g or 78.2% of the original holocellulose. This residue was labeled "Residue B" (55).

The filtrate and combined washings were condensed to 1.0 liters and acidified with acetic acid to pH 5.0. The solids were precipitated by the addition of 3 liters of methanol, and then recovered by centrifuge. The precipitate was redispersed in 700 ml of 70% methanol. This redispersion purification procedure was repeated two more times. The methanol was removed from the final precipitate on a rotary evaporator. The solids were slurried in water and the mixture was freeze-dried. The weight of solids recovered was 0.84 g or 1.7% of the original holocellulose. These solids were labeled "Hemicellulose B".

3. Extraction with 15.0% Aqueous Sodium Hydroxide Solution

To "Residue B" (38.6 g dry weight) was added 923 g of a 15.05% aqueous sodium hydroxide solution to give a 4.0% slurry consistency based on the content of Residue B and a solution concentration of 15.0% in sodium hydroxide. After stirring for 20 min at 25°, the insolubles were recovered by filtration using milk filter disks. The residue was washed with 250 ml of 15.0% aqueous sodium hydroxide solution and 1.0 liters of distilled water. The residue was dialyzed for 5 days against running water and freeze-dried; weight 31.3 or 62.6% of the original holocellulose. The residue was labeled "Residue C" (55).

The filtrate and washings were combined, acidified with acetic acid, condensed to 1.0 liters and the solids were precipitated by the addition of 3 liters of methanol. The precipitate was redissolved in alkali and redispersed three times in 700 ml of 70% methanol. The methanol was removed on a rotary evaporator and the precipitate freeze-dried; weight 1.46 g or 2.9% of the starting holocellulose. These solids were labeled "Hemicellulose C". The rotation was taken in 1 N sodium hydroxide solution, $[\alpha]_D^{23.5}$ - 40° (c 0.25, N sodium hydroxide).

K. Characterization of Hemicellulose A; Isolation of a Xylan

1. Qualitative Carbohydrate Analysis by Paper Chromatography

Hemicellulose A (262.2 mg dry weight) was dissolved in 4.5 g of 77% sulfuric acid at ice-bath temperature in a 150.0 ml round-bottomed three-necked flask. The mixture was stirred until complete dissolution of Hemicellulose A was achieved (1.0 hr). The translucent sirup was diluted to 3.9% sulfuric acid by the addition of water (84.42 ml). The dilute solution was refluxed, neutralized, and paper chromatographed as described above for holocellulose hydrolysis. Paper chromatograms of the hemicellulose A hydrolyzate showed large amounts of xylose, minor amounts of glucose, galactose, and arabinose and trace amounts of mannose. Hemicellulose A was thus considered to be a "xylan".

The isolation procedure outlined in section J-1 was repeated and a large amount of the crude xylan was isolated.

2. Ash Determination of the Crude Xylan

The ash content of the crude xylan was determined according to a modified TAPPI standard method (56).

Duplicate samples (0.2 g dry weight) were weighed into previously ignited and weighed porcelain crucibles. The crucibles were placed in a muffle furnace and heated gently (200°) until charring occurred. The temperature was increased to 600° and heating continued until ashing was complete. The ashed samples were placed in a dessicator to cool and were weighed after 1.0 hr. The samples were then reignited and reweighed until constant weight was obtained. The ash content was 27.9%.

3. Precipitation of Excess Barium and Dialysis of the Crude Xylan

For purposes of calculation, it was assumed that the ash content of the crude xylan was due entirely to the presence of barium as barium oxide. From this value, the percent barium (25.0%) present in the sample was calculated. The large barium residue was due to the use of barium hydroxide in the isolation of the crude xylan.

A part (5.06 g) of the crude xylan was dissolved in distilled water (100.0 ml). A slight excess of 2 M sulfuric acid (5.0 ml) was added to precipitate all of the barium in the sample. The precipitate was allowed to settle, recovered by filtration and washed several times with hot distilled water (51, p. 134). The washings and the filtrate were recombined, placed in a dialysis bag and dialyzed against tap water for 5 days. The material left in the dialysis bag was concentrated on a rotary evaporator and freeze-dried (yield,69.8%). A portion of the freeze-dried material was ignited to determine its ash content (4.4%). This purified, freeze-dried material was labeled "xylan".

4. Optical Rotation of the Xylan

A part (0.18235 g) of the xylan was dissolved in water (10.0 ml). Ten different readings were taken on the polarimeter (Rudolf and Sons) at 25°. The specific rotation was calculated according to the relationship (57, p. 415):

$$[\alpha]_D^{25} = \frac{100\alpha}{bc} = \frac{100\alpha}{c}$$

where $[\alpha]_D^{25}$ = specific rotation

α = optical rotation in degrees

b = cell length in dm = 1

c = concentration in g/100 ml

The specific rotation was -30.5, (c 1.74 g/100 ml, water) (average of 10 readings).

5. Qualitative Uronic Acid Analysis by Color Reaction

Concentrated sulfuric acid (6.0 ml) was added slowly to an aqueous solution (1.0 ml) of the 3.0% sulfuric acid hydrolyzate of xylan and cooled under tap water. The reaction mixture was heated for 20 minutes in a boiling water bath and cooled. An aliquot (0.2 ml) of a 0.1% ethanolic solution of carbazole was added and the test sample allowed to stand for 2 hr at room temperature. The development of a purple color indicated that the xylan contained a uronic acid. The solution was scanned in the visible region and showed maximum absorption at 535 nm indicating the presence of a uronic acid (38, p. 497). Color tests were also run on authentic galacturonic acid (positive test), oxalic acid (negative test), and benzoic acid (negative test).

6. Paper Chromatography and Gas-Liquid Chromatography of the Xylan Hydrolyzate

A part (0.30 g) of the dialyzed xylan was hydrolyzed in 3.0% sulfuric acid (mild acid hydrolysis) for 5 hours, cooled to room temperature, neutralized to pH 5.0 with saturated aqueous barium hydroxide and centrifuged to remove the barium sulfate precipitate. An aliquot of the supernatant liquor was concentrated under vacuum in a rotary evaporator. The concentrated liquor was spotted on Whatman No. 1 filter paper. A solution of known sugars was also spotted on the same paper. The paper chromatogram was developed and sprayed as described previously. The sugars detected were xylose (strong), arabinose (weak), galactose (trace), glucose (trace), and rhamnose (trace).

Another aliquot (25.0 ml) of the hydrolyzate was reduced with sodium borohydride and alditol acetates were prepared as described previously. The alditol acetates were injected into a Hewlett Packard Gas Chromatograph using the same column and conditions as described previously. The sugars detected were the same as those reported above for paper chromatography. The areas under the peaks were determined by planimetry.

7. Reducing End-Group Analysis (Somogyi Method)

A Somogyi determination of the reducing end group was carried out on a portion of the dialyzed xylan. The alkaline copper reagent (1945) was prepared as follows: Rochelle salt (40.0 g), disodium hydrogen phosphate dodecahydrate (71.0 g) (or 53.0 g of the heptahydrate), and 1 N sodium hydroxide (100.0 ml). A solution containing cupric sulfate pentahydrate (8.0 g) was added with stirring followed by a solution of potassium iodate (0.372 g in 100 ml water). This amount of potassium iodate is good for determining up to 1.25 mg glucose. Finally, anhydrous sodium sulfate (180.0 g) was dissolved; the solution was diluted to 1.0 liter and allowed to stand 3 days so that the impurities would settle out. The clear supernatant solution was further clarified by filtration through a fritted glass filter. The pH of the solution was about 9.5 as reported in the directions for preparation. The solution is supposed to be stable for at least one year (52, p. 383).

A sample 8.40 mg of the unhydrolyzed xylan in solution (5.0 ml) was placed in a 25 x 200 mm test tube. Alkaline copper reagent (5.0 ml) was added by pipette and mixed thoroughly. The tube was closed with a glass bulb and placed in a rack. Blanks were prepared with water (5.0 ml). Standard xylose solutions (1.25 mg, 0.75 mg and 0.25 mg) were also added to aliquots (5.0 ml) of the alkaline copper reagent. The rack of tubes was immersed in a vigorously boiling water bath to a depth of about 5.0 cm above the solution inside the tubes. The solutions were heated for 30 minutes, removed from the bath and allowed to cool. Care was taken that the tubes were not agitated during the heating or cooling periods. Triplicate determinations were carried out in all cases.

Potassium iodide (2.5%, 2.0 ml) was added to each tube without mixing. From a fast flowing buret, 2 N sulfuric acid (1.5 ml) was run into each tube with shaking so that the liberated iodine would oxidize all reduced copper. After 5 minutes, the tubes were reshaken. The excess of liberated iodine not reduced by cuprous ions was then titrated with sodium thiosulfate (0.005 N). When the solution was light yellow, two drops of starch indicator (1.0%) and two drops of phenol red indicator were added, and the titration continued until the starch-iodine blue disappeared. The difference in the amount of titer consumed by the blank and the xylan was attributed to the reducing end group of the polysaccharide.

8. Viscosity Measurements

A part (0.19156) of the dialyzed xylan was weighed into a stoppered volumetric flask (25.0 ml) which had been previously swept free of dry air by a stream of nitrogen. Diethylenediamine copper II ion (cupriethylenediamine) (General Chemical Division, Allied Chemical, Columbia Road and Park Ave., Morristown, NJ or Ecusta Paper Division, Olin, P.O. Box 200, Pisgah Forest, NC) at 25° was added and the solution was shaken until the xylan was completely dissolved. The flask was filled to the mark with the solvent and mixed thoroughly. The filled flask was carefully weighed to determine the density of the solution in g/25 ml. An aliquot (10.0 ml) of the solution was transferred to a Cannon-Ubbelhode dilution viscometer previously placed in a water bath at 25°±0.01° and flushed with nitrogen. After 5 minutes the solution was drawn into the bulb of the viscometer by applying pressure with nitrogen. The time for the miniscus to pass between the two calibration marks was measured to 0.1 seconds. Duplicate measurements were made until they agreed to within ±0.3% (58, p. 537, 59, 60).

The solution was diluted directly in the viscosimeter with a suitable amount of solvent and mixed by stirring with a stream of nitrogen. Measurements were taken with each dilution. A total of six concentrations were measured. The viscosity of the pure solvent was also measured. The intrinsic viscosity was found to be 0.42 dl/g in diethylenediamine copper II ion.

The intrinsic viscosity was similarly determined in [illegible] aqueous sodium chloride (0.48 dl/g) and in distilled water (0.84 dl/g).

9. Gel Permeation Chromatography - Determination of Molecular Weight Distribution

A chromaflex column (Kontes Glass Co., Vineland, NJ) 400 x 1000 mm with a total bed volume (V_t) of 1250.0 ml was used for the determination of the molecular weight distribution of the xylan.

Dry sephadex G-75 (40-120 μ) was stirred into a 4-liter beaker with distilled water and swollen for 30 hours. During this time the suspension was decanted four times to remove the fine particles. The suspension was well stirred and was transferred through a funnel into the vertically mounted column which had previously been filled with water. After a layer of a few centimeters had formed, the capillary outlet at the bottom of the tube was opened to release a slow stream of water. When all of the gel had settled, the eluant was allowed to flow through the bed overnight to complete the stabilization of the column (40). The void volume (V_o) of 369 ml was determined by use of Blue Dextran 2000 (M_w 2,000,000).

A part (500 mg) of the dialyzed xylan was dissolved in water in a 50.0 ml volumetric flask. After the xylan had completely dissolved the solution was diluted to the 50 ml mark and mixed thoroughly. The xylan solution (20.0 ml) was added dropwise to the top of the column with great care and when it had disappeared below the surface a small quantity of water was applied to wash the surface. Then more water was added to a height of 2-3 cm to start the elution. The elution was carried out under a low hydrostatic pressure at a flow rate of 0.5-0.7 ml/min. The concentration of the eluted fractions (10.0 ml each) was estimated by the phenol-sulfuric acid method to be described later.

10. Acidity of the Xylan

Three samples (49.90 mg, 81,76 mg, 133.43 mg) of the xylan were each dissolved in separate aliquots (10.0 ml each) of distilled water. An aliquot (10.0 ml) of aqueous sodium hydroxide solution (0.0526 N) was added to each of the three samples. Each solution was back titrated to a methyl red end point with hydrochloric acid solution (0.0406 N). Three blank solutions were similarly titrated. The differences between the titers of the blanks and the titers of the xylan samples in the order of the weights given above were: 0.15 ml, 0.25 ml, 0.35 ml.

11. Analysis of Periodate Consumed

A portion (0.26959 g) of the dialyzed xylan was placed in a volumetric flask (50.0 ml), dissolved in water (10.0 ml), after which, 0.5 M sodium periodate solution (25.0 ml) was added and finally water was added to bring the volume up to the 50.0 ml mark. The solution was mixed thoroughly. A blank having the same periodate concentration was also prepared. Both reaction mixtures were incubated in a constant temperature bath at 5° in the dark (61). The time at which the periodate was added was taken as time zero. Aliquots (1.0 ml) of each reaction mixture were withdrawn at timed intervals and added to 250.0 ml iodine flasks containing 1 N sulfuric acid (5.0 ml) and 20.0% potassium iodide solution (5.0 ml). The liberated iodine was titrated with 0.1 N sodium thiosulfate solution until only a pale yellow color remained. Starch indicator (1.0 ml, 1% solution) was added and the titration continued until the blue color disappeared.

12. Analysis of Formic Acid Released

When the consumption of periodate had stopped, aliquots (5.0 ml) of each reaction mixture were added to Florence flasks (150.0 ml) containing ethylene glycol (1.0 ml). The mixture was allowed to stand at room temperature for 5 minutes with occasional swirling until the unreacted periodate was consumed. The solution was titrated with 0.05 N sodium hydroxide solution using methyl red as the indicator.

13. Complete hydrolysis of the Polyalcohol

The oxidized xylan from the periodate reaction was placed in a dialysis bag and dialyzed against running tap water for two days. At the end of this period, it was taken out and concentrated under vacuum in a rotary evaporator to about 100.0 ml. The concentrate was neutral to litmus paper.

An excess of sodium borohydride (1.5 g) was added to the concentrate and the reduction was allowed to proceed at room temperature for 24 hours with occasional shaking. At the end of the reduction period the reaction mixture was returned to the dialysis bag and dialyzed against running tap water for 3 additional days (100).

The polyalcohol was hydrolyzed under mild acid hydrolysis conditions as described previously. The hydrolyzate was spotted on Whatman No. 1 filter paper and paper chromatographed. The sugars in another aliquot (25.0 ml) of the hydrolyzate were converted to their alditol acetates and analyzed by gas-liquid chromatography as described previously. The spectrum showed xylitol pentaacetate and arabinitol pentaacetate and three small unidentified peaks which came out early. The areas under the peaks were measured with a planimeter.

14. Degradation of the Xylan in Sodium Hydroxide Solution

A part (4.34 g) of the dialyzed xylan was placed in a volumetric flask (25.0 ml), which had previously been evacuated and flushed with nitrogen, and dissolved in sodium hydroxide solution (1.0 N). After the xylan was completely dissolved the solution was diluted to the mark with sodium hydroxide solution and shaken vigorously to attain complete mixing. The time when the sodium hydroxide was added was recorded.

Glass ampules (5.0 ml) were evacuated and filled with nitrogen in a glove box. Aliquots (1.0 ml each) of the xylan solution were placed in the ampules. The ampules were taken out of the glove box, sealed, and placed in boiling water (62, 63). The ampules were withdrawn from the constant temperature bath at definite time intervals (30, 60, 90, 120, 180 min and so on). Each withdrawn ampule was allowed to cool for 1 to 2 min and placed in ice water to stop the reaction. Normal hydrochloric acid (1.0 ml) was added to the contents of the ampule to neutralize the sodium hydroxide reaction mixture.

The degradation was also carried out in sodium hydroxide solutions of 0.001 N, 0.01 N, 0.1 N, 4 N and 6 N concentration.

15. Phenol-Sulfuric Acid Method of Analysis of the Xylan

The analysis for the undegraded xylan was carried out by the phenol-sulfuric acid method of Smith and coworkers (64, 65). A Beckman Model DB spectrophotometer was used for this investigation.

Reagent grade sulfuric acid (95.5%, specific gravity 1.84) conforming to ACS specifications was used. Phenol (80% by weight) was prepared by adding glass distilled water (20.0 g) to redistilled reagent grade phenol (80.0 g). This mixture formed a water-white liquid which was readily pipetted and has been known to stay the same color after one year of storage (30). A pale yellow color developed after several weeks but this did not interfere in the determination since a blank was included.

A fast delivery pipette (5.0 ml) was used to deliver the concentrated sulfuric acid. The pipette was prepared by cutting a portion off the tip of a standard 5.0 ml pipette. A high maximum temperature was desired because it increased the sensitivity of the reagent.

Water (1.7 ml) and 80% phenol (0.05 ml) were added to an aliquot (0.3 ml) of the neutral xylan solution in a test tube. Concentrated sulfuric acid (5.0 ml) was added rapidly. The stream of acid was directed against the liquid surface in order to obtain good mixing. Intense heat was generated at this time. The tubes were allowed to stand for 10 minutes, then shaken and placed in a water bath at 25 to 30° for another 10 to 15 min. The test tubes were shaken again and samples were transferred to spectrophotometer cells (1.0 cm light path) and readings taken at 480 nm. The color developed was stable for several hours. The weight of hydrolyzed pentoses and uronic acids was expressed as xylose equivalents by using percent transmission in conjunction with a standard curve prepared from known xylose.

L. Characterization of Hemicellulose B; Isolation of a Glucomannan

1. Qualitative Carbohydrate Analysis by Paper Chromatography

Hemicellulose B (250.8 mg, dry weight) was dissolved in 4.5 g of 77% sulfuric aicd for 1.0 hr at ice-bath temperature. The resulting translucent solution was diluted to 3.9% sulfuric acid by the addition of 84.42 ml of distilled water. The dilute solution was refluxed, neutralized, and paper chromatographed as described above for holocellulose hydrolysis. Paper chromatograms of the Hemicellulose B hydrolyzate showed large amounts of glucose and mannose, minor amounts of galactose and xylose, and trace amounts of arabinose.

M. Characterization of Hemicellulose C; Isolation of a Mannan

1. Qualitative Carbohydrate Analysis by Paper Chromatography

Hemicellulose C (273.3 mg, dry weight) was hydrolyzed, neutralized, and paper chromatographed as described above for Hemicelluloses A and B. The Hemicellulose C hydrolyzate contained large amounts of mannose, small amounts of glucose and galactose, and trace amounts of arabinose and xylose.

2. Quantitative Carbohydrate Analysis by Gas-Liquid Chromatography

A portion of Hemicellulose C (150 mg, air-dry weight, moisture content 8.9%) was acid hydrolyzed, neutralized, reduced and acetylated as described previously for the quantitative carbohydrate analysis of the holocellulose fraction by gas-liquid chromatography. An aliquot (20 mg) of the resulting alditol acetate sirup was dissolved in methylene chloride and diluted to the mark in a 1.0 ml volumetric flask. An aliquot (2 μl) of the solution was injected into the gas chromatograph under the general conditions previously established. The areas under the peaks of the resulting spectrum were measured by a planimeter.

3. Methylation of the Mannan

A portion of Hemicellulose C (1.4 g, air-dry weight, moisture content 8.9%) was dissolved in 18% aqueous sodium hydroxide solution (50.0 ml) at ice-bath temperature with stirring. The solution was methylated by the simultaneous addition of 30% aqueous sodium hydroxide solution (100.0 ml) and dimethyl sulfate (50.0 ml) over a period of 3 hr while maintaining the solution at ice-bath temperature. Acetone (100.0 ml) was added to prevent foaming. The mixture was stirred for an additional 45 hr at room temperature. The solution was cooled to 0° in an ice-bath, neutralized with 10% sulfuric acid, and dialyzed for 3 days against running tap water. The solution was concentrated on a rotary evaporator, freeze-dried and the entire methylation sequence was repeated (41).

The product was further methylated by dissolution in tetrahydrofuran (150.0 ml) followed by treatment over a period of 60 hr with seven 10.0 g portions of crushed sodium hydroxide, each followed by a portion (12.0 ml) of dimethyl sulfate (42). The product was dialyzed against running tap water for 3 days. The concentrated dialyzate was extracted with three 300.0-ml portions of chloroform. The chloroform extract was concentrated on a rotary evaporator, dissolved in acetone and filtered. The concentrated filtrate (50.0 ml) was poured into petroleum ether (200.0 ml), and the white precipitate which resulted was recovered and freeze-dried; yield of a white powder 40.7 mg. An infrared spectrum (potassium bromide pellet) of a portion of the methylated Hemicellulose C showed no absorption in the regions of 3200 cm^{-1} or 1750 cm^{-1} indicating that there were no free hydroxyl groups in the material. Therefore, methylation was considered complete.

4. Hydrolysis of the Methylated Mannan

The methylated mannan (40.7 mg) was hydrolyzed with 10.0 ml of 90% formic acid at 97° for 3 hr. The formic acid was removed by evaporation under reduced pressure followed by the addition and removal of water. The sirup so obtained was hydrolyzed with 0.5 N sulfuric acid (5.0 ml, for 2.5 hr at 97°). Upon cooling, the solution was neutralized with barium hydroxide, and the solids were removed by centrifugation. The remaining inorganic salts were removed by refluxing the concentrated sirup with absolute methanol, followed by filtration (repeated four times) and removal of solvent (41).

The sirup so obtained was dissolved in a few drops of water and subjected to paper chromatography using 2-butanone saturated with water as the developer (67). The chromatogram was allowed to run until the solvent front had moved 32 cm from the origin where the sample was applied (2.5 hr). The developed chromatogram was allowed to air-dry and then sprayed with aniline phthalate indicator (1.66 g of phthalic acid dissolved in 100.0 ml of water-saturated _n_-butanol containing 0.93 g of freshly distilled aniline) (68, 69). The papers were allowed to air-dry again for 15 min, heated in an oven at 105° for 15 min and viewed under ultraviolet light. Three spots were evident with the following R_f values: 0.89 (trace), 0.51 (very strong), 0.24 (trace). (R_f value is the ratio of the distance the spot moved divided by the distance the solvent front moved, both measured from the point of application of the material).

A second aliquot of the hydrolyzate of the methylated mannan was subjected to paper chromatography using *n*-butanol-ethanol-water (5:1:4 v/v/v) as developer. The solvent front was allowed to migrate 40 cm past the origin where the spot was applied (19 hr). The papers were sprayed with aniline phthalate indicator as described above. Seven spots were evident which had the following R_G values (R_G values determined by dividing the distance the sugars have moved from the starting line by the distance moved by 2, 3, 4, 6-tetra-*O*-methyl-*D*-glucopyranose): 0.96 (trace), 0.82 (very strong), 0.64 (trace), 0.52 (trace), 0.38 (trace), 0.28 (trace), 0.21 (trace).

5. Molecular Weight Determination of the Mannan by Viscosity Measurements

An amount (125 mg, dry weight) of the mannan (Hemicellulose C) was weighed into a 25.0 ml volumetric flask which had been previously weighed and swept free of air by a stream of nitrogen gas. Diethylenediamine copper II reagent at 25° was added and the mixture shaken. However, not all of the mannan dissolved. The mixture was transferred to a 100 ml volumetric flask and diluted with additional diethylenediamine copper II reagent. However, the mannan still did not completely dissolve.

N. Characterization of Residue C; Isolation of a Glucan

1. Hydrolysis with 77.0% Sulfuric Acid

A portion (200 mg, air-dry weight, moisture content 11.1%) of the residue from the 15.0% aqueous sodium hydroxide extraction (Residue C) was hydrolyzed with 77.0% sulfuric acid according to the procedure previously described.

Paper chromatography of the hydrolyzate showed the presence of a large amount of glucose, medium amounts of mannose and galactose, and trace amounts of xylose and arabinose.

2. Four Hour Formic Acid Extraction

A portion (1.0 g, air-dry weight) of Residue C remaining after the 15.0% aqueous sodium hydroxide extraction was refluxed (97°) with 100.0 ml of 95%

formic acid for 4 hr. The insoluble residue was recovered by centrifuge, washed with water and freeze-dried; weight 0.18 g.

A portion (0.15 g) of the above residue was hydrolyzed with 77.0% sulfuric acid and the hydrolyzate paper chromatographed as previously described. The chromatograms showed a large amount of glucose, a small amount of mannose, and trace amounts of arabinose and xylose. Thus the formic acid did not completely remove the non-glucose containing polysaccharides.

3. Four Day Formic Acid Extraction

A portion (10.0 g) of Residue C was extracted with 200.0 ml of 95% formic acid for 4 days in a Soxhlet extractor using a porcelain extraction thimble. An amount (25.0 ml) of additional formic acid was added each day. At the end of 4 days the residue was recovered by washing with distilled water, centrifuged, and freeze-dried; weight 3.6 g.

An amount (150 mg, air-dry weight, moisture content 11.1%) of the above residue was hydrolyzed with 77.0% sulfuric acid and the hydrolyzate was paper chromatographed as previously described. The paper chromatogram showed glucose only.

4. Quantitative Carbohydrate Analysis by Gas-Liquid Chromatography

An amount (150 mg, air-dry weight, moisture content 11.1%) of the residue from the formic acid extraction was hydrolyzed with 77.0% sulfuric acid as previously described. The hydrolyzate was reduced with sodium borohydride, and the alditols were acetylated and analyzed by gas-liquid chromatography as previously described. The areas under the peaks of the resulting spectra were measured with a planimeter.

5. Acetolysis of the Glucan

An amount (2.0 g air-dry weight, moisture content 11.1%) of the residue from the formic acid extraction was kneaded into a mixture of acetic anhydride

(8.0 ml) and concentrated sulfuric acid (0.2 ml), at ice-bath temperature (3°), until completely wetted. The mixture was placed in an oven at 50° for 14 days (70). The resulting dark brown sirup was mixed with acetic acid (10.0 ml). The suspension was stirred with 500.0 ml of cold water and an additional 250.0 ml of water was added. The mixture was stirred for 30 min and filtered through a sintered-glass funnel. The residue was washed free of acid with water, air-dried, and extracted with boiling ethanol (95%, 200.0 ml).

The aqueous filtrate from the above filtration was extracted with chloroform (250.0, 250,0, 100.0 ml). The chloroform extract was washed with 10.0 ml of a saturated aqueous solution of sodium carbonate and concentrated on a rotary evaporator to yield a solid. However, the material resisted crystallization.

Since no crystals resulted from the above acetolysis, a second acetolysis was attempted simultaneously with the acetolysis of filter paper. The acetolysis of filter paper (2.0 g) resulted in crystals of cellobiose octaacetate (1.56 g), m.p. 228-228.5°, literature value 223-224°.

However, still no crystals resulted from the acetolysis of the residue from the formic acid extraction. Therefore, the reaction was repeated but the reaction time was shortened from two weeks to one week. Crystals of cellobiose octaacetate (550 mg) were obtained. The material was recrystallized three times from 95% ethanol; yield 275 mg, m.p. 224-225°, $[\alpha]_D^{23}$ + 40° (*c* 3.0, chloroform); unchanged on admixture with authentic material, literature value 223-224°, $[\alpha]_D^{22}$ + 40° (*c* 3.0, chloroform).

6. Molecular Weight Determination of the Glucan by Viscosity Measurements

An amount (100 mg, dry weight) of the residue from the formic acid extraction was placed into a 25.0 ml stoppered volumetric flask which had been previously weighed and swept free of air by a stream of nitrogen gas. Diethylenediamine copper II reagent (cupric ethylenediamine) (General Chemical Division, Allied Chemical, Columbia Road and Park Ave., Morristown, NJ or Ecustic Paper Division, Olin, P.O. Box 200, Pisgah Forest, NC) at 25° was added and the solution was shaken until the glucan was completely dissolved. The flask was filled to the mark with the solvent and mixed thoroughly. The filled flask was carefully weighed to determine the density of the solution in g/25 ml. An aliquot (10.0 ml) of the solution was transferred to a Cannon-Ubbelhode dilution viscometer previously placed in a water bath at 25±0.01° and flushed with nitrogen. After 5 min the solution was drawn into the belt of the viscometer by applying pressure with nitrogen. The pressure was released and the time required for the miniscus to pass between the two calibration marks was measured to 0.1 seconds. Triplicate measurements were made until duplicates agreed to within ±0.3% (58, p. 537, 59, 60).

The solution was diluted directly in the viscometer with a suitable amount of solvent and mixed by stirring with a stream of nitrogen. Measurements were taken with each dilution. A total of six concentrations were measured. The viscosity of the pure solvent was also measured. The intrinsic viscosity was found to be 0.432 dl/g in diethylenediamine copper II ion.

7. Methylation of the Glucan

A portion (1.4 g, air-dry weight, moisture content 11.1%) of the residue from the formic acid extraction was methylated by the procedure previously outlined for the methylation of Hemicellulose C; yield 605 mg. An infrared spectrum (potassium bromide pellet) of a portion of the methylated material showed no absorption in the regions of 3200 cm^{-1} or 1750 cm^{-1} indicating that there were no free-hydroxyl groups. Therefore, methylation was considered complete.

8. Hydrolysis of the Methylated Glucan

A part of the methylated glucan (150 mg, dry weight) was dissolved in 2.25 g of 77.0% sulfuric acid at the ice-bath temperature for 1 hr and then diluted with 42.2 ml of water to 3.9% sulfuric acid concentration. The solution was refluxed for 6 hr. There remained considerable insoluble materials in the solution which were removed by centrifugation. The decantate was neutralized with saturated aqueous barium hydroxide solution to pH 5.0 and the precipitate was removed by centrifugation. The resultant hydrolyzate was condensed to a sirup on a rotary evaporator.

The sirup so obtained was dissolved in a few drops of water and subjected to paper chromatography using 2-butanone saturated with water as the developer (67). The sirup from the hydrolysis of the methylated mannan was chromatographed on the same sheet of paper. The chromatogram was allowed to run until the solvent front had moved 32 cm from the origin where the sample was applied (2.5 hr). The developed chromatogram was allowed to air-dry and then sprayed with aniline phthalate indicator (1.66 g of phthalic acid dissolved in 100.0 ml of water-saturated n-butanol containing 0.93 g of freshly distilled aniline) (68, 69). The papers were allowed to air-dry again for 15 min, heated in an oven at 105° for 15 min and viewed under ultraviolet light. Four spots were evident from the hydrolyzed glucan with the following R_f values: 0.82 (trace), 0.56 (very strong), 0.21 (trace), 0.06 (trace).

A second aliquot of the hydrolyzate of the methylated glucan and of the methylated mannan were subjected to paper chromatography using n-butanol-ethanol-water (5:1:4 v/v/v) as developer. The solvent front was allowed to migrate 40 cm past the origin where the spot was applied (19 hr). The papers were sprayed with aniline phthalate indicator as described above. Five spots were evident from the hydrolyzate of the methylated glucan with the following R_G values (R_G values determined by dividing the distance the sugars have moved from the starting line by the distance moved by 2, 3, 4, 6-tetra-O-methyl-D-glucopyranose): 1.0 (trace), 0.83 (very strong), 0.67 (trace), 0.41 (trace), and 0.28 (trace).

III. Results and Discussion

A sample of inner bark free from outer bark and cambium was desired because it provided a relatively homogeneous starting material. Outer bark contains considerable cork material which would interfere with experimental studies of the carbohydrates. The cambium layer contains proteins as well as carbohydrates and the two are best separated at the outset.

In the spring of the year from April through June, the outer bark of Douglas-fir is easily separated from the inner bark by simply chipping it away. The inner bark for the present work was taken from a standing tree to reduce contamination from other sources. The cambium layer was then carefully separated from the inner bark and a relatively homogeneous sample resulted.

The inner bark after the ethanol-water extraction was carefully ground, screened and reground to ensure that most of the fibers were separated and that the surface areas were exposed. Some of the fine material was included in the fraction to provide a large starting weight to ensure that sufficient polysaccharides would be isolated for later studies. All of the fines were not included because preliminary experiments showed that they plugged the extraction apparatus and filter systems and made laboratory procedures time-consuming and difficult.

It is desirable, before proceeding with polysaccharide separation, to remove as much as possible of the low-molecular weight materials (simple sugars, organic acids, lipids, waxes, and so on) present in the bark. Some of these materials are readily oxidized and would interfere with the delignification reaction.

Figure 5 outlines the scheme followed in the treatment of the inner bark and the isolation of the different fractions. The yields for each extraction are shown. None of the extraction procedures shown in this chart accomplished a clear-cut separation of one type of material from another, but in general each step in the sequence performed a definite function.

The ethanol-water (4:1 v/v) extraction served two purposes. It prevented possible enzyme action which might change the natural materials from their native state, and it also solubilized the simple sugars which could be identified to provide a more detailed investigation of the total carbohydrate fraction in the bark. The ethanol-water (4:1 v/v) soluble solids were shown to contain the free sugar, glucose, by paper chromatography.

The benzene-ethanol (2:1 v/v) azeotrope removed lipids and waxes which would interfere with later reactions. The extract contained no free sugars when investigated by paper chromatography. Having de-fatted the bark it was possible to carry out a hot water extraction and remove a water-soluble fraction which amounted to 11.1% of the original bark sample.

Compounds isolated from natural sources often contain one or more of the elements, nitrogen, sulfur, phosphorus or the halogens. Therefore, it is best to qualitatively determine if they are present.

The sodium fusion test on the water-soluble fraction failed to detect the presence of sulfur, phosphorus, and the halogens. Nitrogen was shown to be present as evidenced by the formation of a precipitate of Prussian blue with ferrous sulfate.

A portion of the sample was sent to an analytical laboratory (Pascher and Pascher, Mikroanalytisches Laboratorium 53 Bonn, Buschstrasse 54, West Germany) for analysis of nitrogen by the Kjeldahl method, and was found to contain 3.63% nitrogen. A part of this nitrogen is in the form of proteins as discussed later.

A ferric chloride-potassium ferricyanide test produced a dark-blue color. This color (Turnbull's blue) was due to the formation of a 1:1 complex of ferric ion and phenolic hydroxyl which give the ferrous reaction. The iodine test likewise resulted in a blue color characteristic of a starch-iodine complex.

These strongly positive color tests showed the presence of considerable amounts of tannins and starch. Kiefer and Kurth (8) indicated the possibility of phenolic materials in Douglas-fir inner bark and starch has been reported in the hot-water-soluble fractions from several inner barks (63). Therefore, starch was expected to be present in Douglas-fir bark.

A fundamental aspect of polysaccharides is the component monosaccharides which are linked together to form the polymer chain. Often polysaccharides contain linkages which are resistant to acid cleavage. Thus, to ensure complete hydrolysis, the hot-water-soluble solids were treated with strong acid according to the procedure of Laver, Root, Shafizadeh and Lowe (22). A portion of the hot-water-soluble solids would not dissolve in 72.0% sulfuric acid. After hydrolysis, the insoluble portion had a dark-red color and presumably were phenolic acids. This precipitate was not investigated. The hydrolyzate was analyzed for monosaccharides as discussed below.

If the polysaccharides under investigation are water soluble and if hydrolysis can be accomplished under mild acid conditions, treatment with strong acids as discussed above is not desirable because of degradative side reactions. Since the hot-water-extracted solids were water soluble, a mild acid hydrolysis (3.0% sulfuric acid) was compared to the 72.0% sulfuric acid hydrolysis. The hydrolyzate was analyzed for amino acids and monosaccharides as discussed below.

As was the case in the strong acid hydrolysis, the insoluble portion of the extract had a reddish appearance. This precipitate was found to persist later even after purification of the water-soluble solids.

Quantitative analysis showed that the hot-water-soluble solids contained 3.63% nitrogen. Bark has been known to contain both protein and non-protein nitrogen, as well as free amino acids. At least 5 water-soluble proteins have been detected. The cambium layer of Douglas-fir bark has also been shown to contain protein (17), and possibly some cambium layer may have remained with the inner bark during the sample preparation.

The hydrolyzate from the mild acid hydrolysis (3.0% sulfuric acid) of the hot-water-soluble solids was examined for amino acids by two-dimensional paper chromatography.

The chromatograms showed several purple spots but the spots were not well resolved. Lai (62) found that 3.0% sulfuric acid hydrolysis did not appear to be strong enough to hydrolyze the proteins into their component amino acids. The strong acid treatment (72.0% sulfuric acid) better hydrolyzed the proteins into their component amino acids. The evidence in the present work shows that proteins were extracted by the hot-water treatment.

Paper chromatography of the original extract failed to detect free monosaccharides. Therefore, the carbohydrates present in the hot-water-soluble fraction are considered to be in the form of polysaccharides.

The mixture of monosaccharide sugars resulting from acid hydrolysis of the hot-water-soluble solids was well resolved by paper chromatography. The chromatograms showed a very large amount of glucose, a moderate amount of arabinose, a slight amount of galactose, trace amounts of xylose and rhamnose and almost no mannose.

The hydrolyzates from both the strong acid hydrolysis and mild acid hydrolysis contained what appeared to be identical monosaccharides by paper chromatography. Therefore, strong acid hydrolysis was not necessary, and to avoid degradation, the 3.0% sulfuric acid method was used in all subsequent hydrolyses.

The five major sugars, glucose, mannose, galactose, arabinose and xylose are those ordinarily found in wood and wood pulp (20). Rhamnose in Douglas-fir bark was first reported by Lai (62) in the acidified acid hydrolyzate of the sodium chlorite-soluble fraction. Its identification in the acid hydrolyzate of the hot-water-soluble solids supports its finding in the polysaccharides of Douglas-fir inner bark.

Figure 6 outlines the scheme followed for the purification of the polysaccharides extracted by hot water. When the freeze-dried sample was redissolved in distilled water at room temperature, it was found that 29.8% of the sample would not dissolve. The insoluble portion (Fraction A) gave a strong positive reaction to the iodine test showing the presence of starch and a negative test to the ferric chloride-potassium ferricyanide solution showing the absence of phenolics. Strong acid hydrolysis and paper chromatography indicated the presence of glucose only. No traces of other monosaccharides could be detected. Microscopic examination of this sample showed some fiber debris and irregularly shaped materials which were presumably starch granules. Since only glucose was detected and the most obvious sources of this monosaccharide were starch and cellulose, this fraction was not investigated further.

Ethanol was added to the water-soluble portion to provide a solution of 70.0% in ethanol. A precipitate formed which amounted to 25.9% of the original water-soluble solids. These precipitated solids were labeled Fraction B (Figure 6). Those solids which remained soluble in the 70.0% ethanol solution (44.3% of the original water-soluble solids) were labeled Fraction C (Figure 6). Fraction C was found to react strongly with ferric chloride-potassium ferricyanide indicating the presence of phenolics but gave no reaction with iodine indicating the absence of starch. This fraction was not studied further. Polysaccharides which may be in this fraction would be expected to possess a very low molecular weight.

The fraction insoluble in the 70.0% ethanol-water mixture (Fraction B) gave a positive reaction with iodine indicating the presence of starch. It also gave a slightly positive reaction with ferric chloride-potassium ferricyanide, thus indicating that separation of the polysaccharides from the phenolics was not complete.

Hydrolysis of Fraction B and two-dimensional paper chromatography of the hydrolyzate showed the presence of amino acids. Six major spots, seven light spots, five very light spots and two large smears showed on the paper chromatogram. The identification of these amino acids was not investigated further because the main objective of this study was the carbohydrates. However, the paper chromatogram definitely showed the presence of protein in the inner bark of Douglas-fir.

Paper chromatography of the monosaccharides showed the same relative amounts of sugars as found in the whole hot-water-soluble fraction.

Fraction B (Figure 6) was treated with α-amylase enzymes to remove starch. At the end of the hydrolysis the reaction mixture was dialyzed

(Figure 6). The dialyzate showed the presence of glucose only, both before and after hydrolysis with acid. No other monosaccharides were detected.

The enzymes used in this investigation were α-amylases and therefore specific for the hydrolysis of the (1→4)-O-α-D-glucopyranosyl-type bond. The fact that glucose was released proves the presence of an α-D-(1→4)-glucan, undoubtedly the amylose portion of starch. This represents the first time that starch has been shown by chemical analysis to be present in the inner bark of Douglas-fir. The starch undoubtedly acts as a food reserve for the living cells in the inner bark.

The non-dialyzables from the enzyme hydrolysis showed a blue color with iodine indicator. Thus the α-amylases did not hydrolyze all of the starch. This was undoubtedly due to the presence of some amylopectin in the starch molecule. Amylopectin possesses some α-D-(1→6)-glucan branch chains which are not cleaved by α-amylases. The residue was, therefore, expected to contain some residual starch molecules.

The original hot-water-soluble solids were found to have a nitrogen content of 3.63%. The protein content of natural products is generally calculated as the product of the nitrogen percentage and the factor 6.25, which is the usual factor used for conversion of nitrogen content to protein content. In accordance with this calculation the hot-water-soluble solids of the inner bark of Douglas-fir contained 21.8% protein. This appeared to be a very high protein content, but when it was corrected to the original bark weight (Figure 5) the value was 2.42% protein in the inner bark of Douglas-fir. Values for bark proteins which have been reported are 5.0% for the inner bark of birch and an average of 21.6% for the inner bark of black locust (58, p. 625). Therefore, in comparison, Douglas-fir bark appears quite low in protein content.

The quantitative nitrogen content (Kjeldahl) of Fraction D (Figure 6) was 1.57%. This proved to be a reversal in nitrogen content. The original water-soluble solids contained 3.63% nitrogen as previously mentioned. Extraction with water at room temperature and precipitation with 70% ethanol provided Fraction B (Figure 6) which contained 0.28% nitrogen. Apparently in the re-precipitation of the water-soluble solids, a large amount of the protein remained in solution with the phenolic compounds. In an effort to obtain purer polysaccharide samples, and in particular to remove the starch, Fraction B was treated with starch hydrolyzing enzymes (HT-1000 and Diazyme L30) and with protein hydrolyzing enzymes (chymotrypsin and trypsin; Figure 6). The nitrogen content of the final product (Fraction D) increased to 1.57%. It would appear from this increase in nitrogen content that not all of the enzymes were removed by dialysis. Therefore, the net result of the treatments was to introduce protein impurities into the sample.

Fraction D was hydrolyzed with 3.0% sulfuric acid and paper chromatographed. The paper chromatogram showed a large reduction in the amount of glucose present, relative to the amount of the other monosaccharides. Glucose was still the major monosaccharide present, although the spots for arabinose and galactose were now more prominent relative to the glucose spot. A part of the glucose residues may have come from residual starch that was not destroyed by the enzyme. It was thought, however, that another treatment with the starch enzyme would not completely remove the starch and that it might even prove detrimental to the other polysaccharides that were present. Starch enzyme treatment was carried out at a pH of 3.8-4.2 and at this slightly acidic condition, there was a distinct possibility that acid hydrolysis might destroy the polysaccharides that were the object of the purification process.

Carbohydrates are neither heat resistant nor volatile and so derivatives must be prepared which will volatilize without degradation in order to perform gas-liquid chromatographic analysis. There are numerous derivatives which have been tried but the ones most commonly used today are the "alditol acetates." In the preparation of the alditol acetates the monosaccharides are first treated with sodium borohydride to reduce the aldehyde function to the alcohol function. This has the result of preventing ring isomerization of the pyranose and furanose forms and so prevents the formation of alpha and beta forms of the sugars. The end result is only one form, the alditol, for each of the monosaccharide sugars, rather than three, four or even five isomers. The acetates are synthesized from the alditols to make a volatile, heat resistant derivative.

The gas chromatographic resolutions of the alditol acetates prepared from Fractions B and D (Figure 6) were excellent. The areas under the peaks of the different sugars were compared to each other to give relative weight values. The areas under the peaks of the other sugars were compared to the area of the derivative for galactose which was taken as 1.0. For Fraction B (Figure 6) the areas under the curves were: galactitol hexaacetate, 1.0; arabinitol pentaacetate, 1.4; and glucitol hexaacetate, 32.4. Only traces of rhamnitol pentaacetate (0.1), xylitol pentaacetate and mannitol hexaacetate were found. For Fraction D (Figure 6) the areas were galactitol hexaacetate, 1.0; arabinitol pentaacetate, 1.3; glucitol hexaacetate, 2.9; and traces of rhamnitol pentaacetate, xylitol pentaacetate and mannitol hexaacetate. These relative areas are in agreement with the relative area and intensity of color developed by the spots on the paper chromatograms of both samples.

The starch enzyme treatment resulted in a decrease of glucitol hexaacetate from 32.4 units to 2.9 units when compared with galactitol hexaacetate. The ratio (1.4:1.0) of arabinose to galactose in Fraction B is the same as in Fraction D (1.3:1.0), allowing for errors in measurements. The results show that the starch enzyme was effective in reducing the amount of starch in the sample. The almost constant ratio of arabinose to galactose in Fraction B and Fraction D confirms what the paper chromatograms have already shown, that the starch enzymes acted to hydrolyze the starch only. All other polysaccharides were left intact by the starch enzyme treatment. Therefore, it was concluded that the major carbohydrate component of the hot-water-extract was starch.

The arabinose and galactose monosaccharides released by acid hydrolysis undoubtedly exist in the inner bark as part of L-arabino-D-galactans which are known to be present in the wood of conifers. These polysaccharides are the only major wood glycans that can be isolated in good yield by extraction of wood with water before delignification. Their ease of extraction and their useful qualities as gums have brought them into commercial production marketed as the commercial gum, Stractan.

Since the L-arabino-D-galactans are common to the wood in conifers, it is considered possible that the arabinose and galactose containing polysaccharides isolated in the present work are similar. This represents the first time that possible L-arabino-D-galactan polysaccharides have been reported in Douglas-fir bark. Because of the large volumes of Douglas-fir bark which are waste products, it is possible that the bark could become of interest as a raw-material source for these polymers since they have already been marketed as commercial gums.

Not all of the pectic material of plants is extractable with water. The insoluble part, protopectin (calcium pectate) was removed from the water-insoluble residue of Douglas-fir inner bark by extraction with a

0.5% ammonium oxalate solution (Figure 5). Presumably a cation exchange occurred in which insoluble calcium oxalate was formed along with soluble ammonium pectate. The latter was extracted with the filtrate.

Through all of the extractions the bark retained its characteristic brown color. However, the strong oxidizing conditions of the acidified sodium chlorite reaction bleached the reaction mixture to a pale yellow color. Fumes of yellow gases, undoubtedly chlorine and chlorine dioxide, were visible in the reaction vessel above the mixture. These gases emphasize the necessity of performing the delignification reaction in a fume hood and of bubbling nitrogen through the reaction to sweep away these toxic materials. Fresh sodium chlorite was added to the reaction at one hour intervals rather than all of it at the beginning to prevent an excess of gas formation and to give the reaction time to proceed. The residue from the delignification, termed "holocellulose" was a pale yellow color.

The filtrate was yellowish in color and was dialyzed to remove low-molecular weight impurities and the inorganic salts and ions which resulted from the reaction of the sodium chlorite. The solution which remained in the dialysis bag after several days was almost colorless and the solids which were recovered by lyophilization were white.

The delignification reaction has been developed to solubilize the non-carbohydrate components of plants. Thus the insoluble residue (holocellulose) remaining is relatively pure carbohydrate material. However, the reaction does dissolve some carbohydrates and it is these materials that are part of the subject of this study.

The solubilized solids of the present investigation represented a complex mixture of carbohydrates, lignin-like compounds, tannins, proteins, and so forth. Therefore, as described below, a variety of approaches were used to characterize the carbohydrates in the mixture.

The ash content was 13.39±1.57%, as sulfate. Although a considerable amount of inorganic sodium chlorite had been added to the delignification reaction, this was a high ash content considering that the fraction had been dialyzed. Although it would be of interest to know what elements were present in the ash, no effort was made to determine them in the present work.

In an effort to lower the ash content and remove more of the low-molecular weight materials, the acidified sodium chlorite soluble materials were again dialyzed. More than 22.4% passed through the dialysis membrane. Paper chromatography of the dialyzate showed a relatively large spot for glucose, indicating that carbohydrate substances, probably monosaccharides and short chain oligosaccharides, had passed through the dialysis membrane.

The solution retained in the dialysis bag was made to 70% ethanol, a method often employed for the purification of polysaccharides (15, p. 449), and resulted in the precipitation of a white, flocculent material.

However, the sulfated ash content of the precipitate was 11.88±2.83% indicating that dialysis and precipitation did not lower the ash content of the original material below the experimental error of the ash determination. The inorganic materials were either chemically attached to the polysaccharides, possibly as salts, or were physically wrapped in the polysaccharides so that diffusion through the dialysis bag was restricted.

Because of the fact that carbohydrates which might be important to the present investigation passed through the dialysis bag and might be lost, and that dialysis and precipitation did nothing to lower the ash content, it was concluded that simple purification was not feasible. Therefore, all additional investigations were done on the original material.

The original material showed a positive test for nitrogen. However, quantitative analysis showed only 0.84% nitrogen, an amount not considered to represent an interfering contaminant and no special efforts were made to separate the nitrogenous material.

The tests for sulfur, phosphorus and the halogens were negative.

A fundamental aspect of polysaccharides is the component monosaccharides which are linked together to form the polymer chain. Often polysaccharides contain linkages which are resistant to acid cleavage. Thus, to ensure complete hydrolysis the material was treated with strong acid according to the procedure of Laver, Root Shafizadeh and Lowe (22). The hydrolyzate was analyzed for amino acids and monosaccharides as described below.

If the polysaccharides under investigation are water soluble and if hydrolysis can be accomplished under mild acid conditions, treatment with strong acids as described above is not desirable because of degradative side reactions. Since the material was water soluble, a mild acid hydrolysis (3% sulfuric acid) was compared with the 77% sulfuric acid hydrolysis. The hydrolyzate was analyzed for amino acids and monosaccharides as described below.

Elemental analysis showed the presence of nitrogen. It could possibly be a result of some residual ammonium oxalate from the ammonium oxalate extraction or it could be part of protein material. The cambium layer of Douglas-fir bark has been shown to contain protein and possibly some cambium layer remained with the inner bark in sample preparation.

The hydrolyzates from the strong acid hydrolysis (77% sulfuric acid) and the mild acid hydrolysis (3% sulfuric acid) were examined for amino acids by two-dimensional paper chromatography. It was found that the second solvent, n-butanol-formic acid-water (20:6:5 v/v/v) must be prepared just prior to use otherwise gradual esterification occurred and altered its chromatographic usefulness.

Chromatograms of the hydrolyzate from the mild acid treatment did not show amino acids. However, chromatograms of the hydrolyzate from the strong acid treatment showed ten purple spots. Quantitative analysis showed only 0.84% nitrogen content indicating that the amount of protein material was not great. Therefore, no effort was made to identify the amino acids.

The mixture of monosaccharide sugars resulting from acid hydrolysis was well resolved by paper chromatography. The chromatograms showed that the hydrolyzates contained a large proportion of glucose, a moderate amount of arabinose, slight amounts of mannose and galactose and trace amounts of xylose and rhamnose.

The hydrolyzates from both the strong acid hydrolysis and mild acid hydrolysis contained what appeared to be identical monosaccharides by paper chromatography. Therefore, strong acid was not necessary and to avoid degradation, the 3% sulfuric acid method was used in all subsequent hydrolysis.

The five major sugars, glucose, mannose, galactose, arabinose and xylose are those ordinarily found in wood and wood pulp. The trace amount of rhamnose represents the first time it has been found in Douglas-fir bark although it has been reported in other barks.

Since the monosaccharides resulting from the hydrolysis of bark are the same as those found in wood, it is expected that the polysaccharides of bark are similar to those in wood. If this is so then the major carbohydrate component is cellulose, but it also contains some hemicelluloses such as xylans, glucomannans, glucogalactomannans and so forth. The isolation of the cellulose fraction and an evaluation of its properties and its degree of polymerization will be of future interest.

The gas chromatographic resolution of the alditol acetates prepared from the acid hydrolyzate was excellent. The myo-inositol peak at the end of the spectrum resulted from the addition of an accurately measured amount of myo-inositol as an internal standard. The areas under the peaks of the other sugars in the spectrum are compared to the area under the peak of myo-inositol for quantitative results.

The sample taken (moisture-free, ash-free basis) yielded the following percentages of monosaccharides: glucose, 41.5%; arabinose 8.3%; galactose, 2.7%; mannose, 2.6%; xylose, 0.7%; rhamnose, 0.7%. This total of 56.5% is less than 100% because the material contains organic substances other than carbohydrates.

The acidified sodium chlorite delignification reaction is an oxidation reaction. Therefore, it was anticipated that the primary hydroxyl groups in position six of the hexose containing polysaccharides would most likely be oxidized to uronic acids as demonstrated below. Therefore, several qualitative tests were applied to determine if uronic acids existed. Since uronic acids are known to be present in natural materials, all of the tests were run in direct comparison with the residue from the ammonium oxalate extraction, the material from which the fraction was prepared.

The infrared spectrum of the 0.5% ammonium oxalate insoluble material showed a strong absorption at 1590 cm^{-1}, corresponding to absorption by carboxylate ion. Thus the starting material from which the fraction was prepared contained uronic acids. The fraction isolated showed an absorption band at exactly the same place. However, the treatment with acidified sodium chlorite, although an oxidation reaction, did not appear to increase the uronic acid content, at least not as detected by infrared spectroscopy.

The carbazole-sulfuric acid color reaction is said to be reasonably specific for hexuronic acids. This was verified by testing monosaccharides and oxalic acid and benzoic acid. These compounds showed no reaction, indicating that the simple sugars and carboxylic acid functions other than those in uronic acids did not interfere with the color reaction.

The 3% sulfuric acid hydrolyzate showed a positive purple color, indicating the presence of hexuronic acids. The reactions showed a maximum absorption in the ultraviolet region at 535±1 nm as described for the test.

The color was not particularly strong and it was considered possible that since the sulfuric acid had been neutralized with barium hydroxide, some of the uronic acids had formed barium salts and had precipitated with the barium sulfate. Therefore, a part of the acid hydrolyzate was neutralized with ion exchange resin but the results were the same.

The pale yellow color of the "holocellulose" from the first delignification (Figure 5) reaction indicated incomplete delignification. The holocellulose was therefore treated a second time with acidified sodium chlorite solution. A white holocellulose was obtained in a yield of 30.6% based on the original starting bark sample.

This was a considerable reduction in yield from the 44.3% isolated after the first acidified sodium chlorite treatment. However, no effort was made to bleach the material under milder conditions and it is believed that the second oxidation could be performed under conditions which would provide a white holocellulose with little loss of material.

Organic compounds isolated from natural sources often contain one or more of the elements nitrogen, phosphorus, sulfur or the halogens. Therefore, it is best to qualitatively determine if they are present because purification techniques are usually required when they are found. However, the tests for these elements in the holocellulose fraction were negative. Therefore, plant materials, such as proteins, which contain these elements were not present in the holocellulose fraction.

The separation of the carbohydrate materials from other plant substances, particularly lignin, is difficult and results in a choice between two possibilities. Most of the polymeric phenols can be removed by strong oxidation reactions but in such a reaction the degradation of the desired carbohydrates is extensive. However, when mild oxidative conditions are used, as in the present work, considerable polyphenolic substances are not separated from the carbohydrate (holocellulose) fraction.

It is thus necessary to analyze for these polymeric phenols in order to have a complete knowledge of the holocellulose fraction. The usual analysis is the "Klason lignin" determination which involves the hydrolysis of the carbohydrates with strong (72%) sulfuric acid and weighing the insoluble solids which remain as "Klason lignin." The holocellulose isolated from the inner bark of Douglas-fir was found to contain 3.1% Klason lignin. This is not a particularly high amount of this type of material and so the holocellulose fraction seemed to be quite free of highly polymeric lignin-like molecules.

The digestion of a holocellulose material with 72% sulfuric acid (Klason lignin determination) results in the solubilization of some lignin-like substances which are thus not measured as "Klason lignin." These materials are referred to by the term, "acid-soluble lignin" and they must be determined to obtain a complete analysis of a holocellulose fraction (45).

These materials are best analyzed by ultraviolet absorption. Lignin-like compounds strongly absorb energy at 280 nm and this band has been used for the quantitative determination of acid-soluble lignins. However, the formation of 5-(hydroxylmethyl)-furfural from hexoses and furfural from pentoses formed during the refluxing step in the Klason lignin determination was found to interfere with this determination. Browning and Bublitz (44) showed that interference by these compounds could be minimized by determining the absorbance of the filtrate at two wavelengths.

Using absorptivity values for lignin and for carbohydrate degradation products obtained, respectively, from spruce Brauns lignin and from a synthetic mixture of glucose, xylose, mannose, and glucuronolactone which had been subjected to the hydrolysis conditions of the lignin determination, Browning and Bublitz (44) were able to write the following equations:

$$A_{280} = 0.68\ C_D + 18\ C_L$$

$$A_{215} = 0.15\ C_D + 70\ C_L$$

where A_{280} and A_{215} were the absorbance values of the lignin filtrate, 0.68 and 0.15 the absorptivities of carbohydrate degradation products, 18 and 70 the absorptivities of lignin at 280 and 215 nm, respectively, and C_D and C_L the concentrations in grams/liter of carbohydrate degradation products and of soluble lignin in the filtrate. Goldschmid (45) showed that by solving the simultaneous equations, the following expression for the soluble lignin concentration in the filtrate can be obtained:

$$C_L = \frac{4.53\ A_{215} - A_{280}}{300}$$

In the present work the acid-soluble lignin content of the holocellulose isolated from Douglas-fir inner bark was determined by scanning the filtrate from the Kalson lignin determination over the wavelength range from 320 to 200 nm. Absorption maxima were observed at 280 nm and 210 nm. The 280 nm peak was the usual one observed for lignin compounds and the 210 nm peak was slightly shifted from the 215 nm peak reported by Browning and Bublitz (44). However, a shifting of wavelength due to different materials and different analytical techniques are not uncommon (45).

The absorbances at 280 nm and 210 nm were 2.62 and 3.77 respectively. These values were used to calculate the acid-soluble lignin as follows:

$$C_L = \frac{4.53\ A_{215} - A_{280}}{300}\ g/l = \frac{4.53 \times 3.77 - 2.62}{300}\ g/l = 0.48\ g/l.$$

Using this figure in conjunction with the filtrate volume (1544.0 ml) and the dry weight of the sample (1.8264 g) the value of 4.07% acid-soluble lignin in the holocellulose was calculated. Thus the total lignin content was 3.1% (acid-insoluble) plus 4.1% (acid-soluble) to equal 7.2%.

The amount of acid-soluble lignin thus exceeded the amount of acid-insoluble lignin as is generally the case with holocellulose material (39). Therefore, correcting the lignin content for soluble lignin was essential. Although the absorptivity values for lignin and carbohydrate degradation products may be somewhat uncertain, as stressed by Browning and Bublitz (44), this error is small compared with that resulting from not correcting for soluble lignin at all.

An important step in determining the structures of polysaccharide materials is to acid hydrolyze them to their monosaccharide components. For water-insoluble polysaccharides two steps are usually required. The first is to dissolve the material in a strong acid usually sulfuric, and the second is to dilute the acid with water followed by reflux to hydrolyze the glycosidic bonds. It is important to perform the hydrolysis with complete dissolution of polysaccharides and with minimum decomposition of sugars.

The holocellulose fraction from Douglas-fir inner bark was found to completely dissolve in 77.0% sulfuric acid, the concentration used to dissolve cellulose (22). It was necessary to add water to the solution slowly with strong stirring to prevent local heating which might have caused degradation of the sugars. After dilution to 3.9% sulfuric acid concentration, the solution was refluxed to bring about hydrolysis.

The acid solution was neutralized to pH 5.0 with aqueous barium hydroxide, resulting in a heavy precipitate of barium sulfate. This method of neutralization was preferred because the pH could be controlled easily. A final pH of about 5.0 was desired because monosaccharide solutions should not be allowed to become alkaline. The action of alkali on monosaccharides follows three general courses (22): isomerization, fragmentation, and internal oxidation and reduction. Such reactions interfere with the qualitative and quantitative results of monosaccharide analyses and considerable care was taken to avoid them.

The identification of the monosaccharides released from polysaccharides on acid hydrolysis is fundamental to an understanding of these polymers. Paper chromatography has become the standard way of tentatively identifying the monosaccharides. Several solvent systems are well established and operation of the individual sugars can usually be achieved with relative ease.

Paper chromatograms of the hydrolyzate from the holocellulose fraction of Douglas-fir inner bark showed the presence of glucose (very strong spot), mannose (medium spot), xylose (medium spot), galactose (trace spot), and arabinose (trace spot). These sugars moved the same distance as authentic sugar samples on the same chromatogram.

A trace spot was also evident near the origin. This spot was attributed to the presence of glucuronic acid.

Qualitative paper chromatography provided an indication of the monosaccharides present in the holocellulose hydrolyzate. However, it is possible for two or more sugars to migrate the same distance and so paper chromatography did not unambiguously identify the monosaccharides. The chromatography did not show whether the sugars were of the D or the L configurations and hence did not completely designate the sugars. Therefore, it was considered necessary to isolate crystalline derivatives of the sugars to unambiguously prove their presence and their configurations.

To prepare crystalline derivatives it was necessary to first separate the mixture of monosaccharides into their individual, isolated sirups. Since large amounts of each sirup were not required, the isolation was accomplished by preparative paper chromatography as described above. The only limitation to the procedure was the time required to develop a sufficent number of paper chromatograms to obtain enough milligrams of sirup. Each isolated sirup was reacted with reagents which provided derivatives which crystallized readily. In every case derivatives of authentic monosaccharides were also crystallized for comparison purposes.

Although the diethyl dithioacetal acetate derivatives of many monosaccharides have been reported in the literature (46) not all of them crystallize easily. In the present work only the derivatives from authentic D-galactose and L-arabinose crystallized easily although diethyl-dithioacetal acetate sirups of D-glucose, D-xylose and D-mannose were also prepared.

Because only a few milligrams of each monosaccharide sirup was available from the holocellulose hydrolyzate, only the sirups from the easily crystallized galactose and arabinose fractions were reacted to prepare diethyl dithioacetal acetate materials. In this way crystalline penta-O-acetyl-D-galactose diethyl dithioacetal and crystalline tetra-O-acetyl-L-arabinose diethyl dithioacetal were obtained. These materials proved the presence of D-galactose and L-arabinose residues in the polysaccharides from Douglas-fir inner bark.

The acetylation of glucose is a common reaction and in the present work white crystals of penta-O-acetyl-β-D-glucopyranose were readily prepared from authentic D-glucose. The glucose sirup isolated from the holocellulose hydrolyzate also yielded penta-O-acetyl-β-D-glucopyranose upon acetylation. This derivative proved the presence of D-glucose residues in the polysaccharide from Douglas-fir inner bark.

The acetates and the diethyl dithioacetal acetate derivatives of xylose do not crystallize easily. However, D-xylose can be easily identified as its di-O-benzylidene dimethyl acetal and in the present work di-O-benzylidene-D-xylose dimethyl acetal was readily obtained from authentic D-xylose. The xylose sirup isolated from the holocellulose hydrolyzate also yielded di-O-benzylidene-D-xylose dimethyl acetal upon reaction. This derivative proved the presence of D-xylose residues in the polysaccharides from Douglas-fir inner bark.

Mannose is a difficult sugar to crystallize and there are also few derivatives of mannose that crystallize easily. However, D-mannose phenylhydrazone crystallizes readily and can be used to identify and to isolate the sugar. The reaction must be carefully done, or a second molecule of phenylhydrazine will add to the sugar and the phenylosazone will result. In fact, this is what occurs with the other monosaccharides. Phenylosazones cannot be used to identify sugars because both C1 and C2 are involved and sugars which differ only about C1 and C2, such as mannose, glucose, and fructose, cannot be differentiated. Thus to identify mannose, the reaction must be stopped after the addition of only one mole of phenylhydrazine to give mannose phenylhydrazone. Isbell and Frush (49) have reported the reaction conditions to perform this step.

In the present work, D-mannose phenylhydrazone was readily prepared from authentic D-mannose. Because D-mannose phenylhydrazone crystallizes so readily from the reaction system outlined by Isbell and Frush (49), it can be preferentially crystallized from mixtures of monosaccharides. Thus, in the present work, a complete hydrolyzate sirup (77.0% sulfuric acid hydrolysis) of the holocellulose from Douglas-fir bark was reacted with phenylhydrazine. After standing overnight in the refrigerator, white crystals of D-mannose phenylhydrazone were obtained. This derivative proved the presence of D-mannose residues in the polysaccharides from Douglas-fir bark.

There are numerous modifications of the standard Fehling's copper reduction test for the presence of reducing sugars. However, one of the more common methods for the quantitative analysis of reducing sugars is the Somogyi (52) procedure. This method is based on the ability of certain sugars (reducing sugars) to act as reducing agents. The sugar reacts with Cu^{++} in the aqueous alkaline medium to produce cuprous oxide. The cuprous oxide is oxidized by iodine back to Cu^{++} and the excess iodine is titrated with thiosulfate. The reactions are:

$$RCHO + 2\ Cu^{++} + 5\ OH^- \rightarrow RCO_2^- + Cu_2O + 3\ H_2O$$

$$IO_3^- + 5\ I^- + 6\ H^+ \rightarrow 3\ I_2 + 3\ H_2O$$

$$Cu_2O + 2\ H^+ + I_2 \rightarrow 2\ Cu^{++} + 2\ I^- + H_2O$$

$I_2 + 2\ S_2O_3^{--} \rightarrow 2\ I^- + S_4O_6^{--}$

This method has been widely and successfully applied on both milligram and microgram quantities, both titrimetrically and colorimetrically. Its accuracy over a wide range of sugar concentrations, the ease and rapidity of operation, and its proven reliability place it above other micro-oxidation methods (52).

As evidenced from the above equations, thiosulfate anion is used as the standard titer to measure the excess iodine concentration. In the present work the sodium thiosulfate solution was standardized by titration to a starch end-point of an accurately diluted solution of potassium iodate which had been prepared from recrystallized and dried potassium iodate solids.

Several different alkaline copper reagents have been recommended for use in conjunction with the iodometric determination of reduced copper. The most common ones are Somogyi's 1945 (52) reagent and his 1952 reagent. Somogyi's 1945 phosphate-buffered reagent possesses an advantage over the 1952 carbonate-buffered reagent, in that amyloses are held in solution in the 1945 reagent but are precipitated from the 1952 reagent. Therefore, the 1945 reagent has become the standard procedure and was the method of choice in the present work. The amount of potassium iodate added to the reagent determines the amount of iodine released and hence the amount of sodium thiosulfate titer needed. Therefore, some preliminary titrations are often required to determine the best amount of potassium iodate to add. The blank should require between 9.0-10.0 ml of 0.005 M sodium thiosulfate solution. When performed properly the method is very good for determining the overall reducing power of a solution and the precision is ±0.01 mg for D-glucose or about ±2%, averaged throughout the range 0.3 to 3.0 mg of glucose.

When determining the quantitative amounts of monosaccharides released from a holocellulose fraction on acid hydrolysis, it is important that the time of refluxing be carefully determined. If the refluxing time is too short, complete hydrolysis is not realized and the quantitative analysis will be erroneous because those monosaccharide residues linked by the more acid-resistant glycosidic bonds will not be determined. If the refluxing time is too long, acid degradation of the hydrolyzed monosaccharides will be extensive. Acid reversion to new, complicated polymers may also occur on extended heating. The importance of the correct reflux time to achieve maximum monosaccharide yield has been demonstrated by Laver, Root, Shafizadeh and Lowe (22) in their studies of pulp analyses.

The simpliest way to measure the proper time to reflux the acid solution is to measure the reducing power of the hydrolysis solution as the reaction progresses. In the present work this was accomplished by removing aliquots of the refluxing solution at specific times and determining the reducing power of the aliquots by the Somogyi method. Glucose was used as a convenient standard reducing sugar in which terms the concentration of monosaccharides was measured. A plot of the reducing power in glucose equivalents versus the time of reflux showed clearly that 12 hours is the optimum time to reflux the hydrolysis solution to achieve maximum yield of monosaccharides.

The reflux time of 12 hours was considerably longer than the 4.5 hours of reflux time which Laver, Root, Shafizadeh and Lowe (22) showed to be necessary for wood pulps. Therefore, the holocellulose fraction from Douglas-fir inner bark contained glycosidic bonds which were more acid resistant than those in wood pulps. These acid resistant bonds were attributed to the mannose containing polysaccharides in the holocellulose.

Carbohydrates are neither heat resistant nor volatile and so derivatives must be prepared which will volatilize without degradation in order to perform gas-liquid chromatographic analyses.

The derivatives most commonly used today are the "alditol acetates." In the preparation of the alditol acetates the monosaccharides are first treated with sodium borohydride to reduce the aldehyde function to the alcohol function. This has the result of preventing ring isomerization to the pyranose and furanose forms and so prevents the formation of alpha and beta forms of the sugars. The end result is only one form, the alditol, for each of the monosaccharide sugars rather than three, four or even five forms. The acetate esters are synthesized from the alditols to make volatile, heat resistant derivatives.

Identification of compounds by gas-liquid chromatography is realized by comparing the time required for the unknown material to pass through the column with the time required for a sample of the authentic material to pass through the column. These times are called "retention times." Qualitative paper chromatography of the hydrolyzate of the Douglas-fir holocellulose had shown the presence of glucose, mannose, galactose, xylose, and arabinose. Therefore, authentic crystalline alditol acetates of each of these sugars were synthesized to positively determine retention times and instrument response for quantitative analyses. _myo_-Inositol hexaacetate was also synthesized to be used as an internal standard. Each of these crystalline materials was passed through the gas chromatograph and the retention time for each was determined. The crystalline materials were then mixed and again passed through the gas chromatograph to ascertain the resolution. The separation of each alditol acetate was excellent and the retention times of each was measured at the center of the peak.

There are many parameters which can be varied in gas-liquid chromatographic techniques. The resolution of the peaks will be changed by changes in the carrier gas flow and changes in the column temperature. Peak heights and peak areas will also change with changes in injection port and detector temperatures. Therefore, in the present study several conditions were systematically changed until the optimum conditions were determined. The conditions used throughout the rest of the work were: column, 6.5% ECNSS-M on Gas Chrom Q 100/120 mesh, 6 ft x 1/8 in O.D. stainless steel; injection port 180°; detector 240°; column temperature 180° isothermal; helium flow 30 ml/min; range setting 10^2; attenuation setting 16.

Gas chromatographic detectors respond differently to different compounds. These response factors must be known to obtain quantitative results. The recorder is also a possible source of error and the precision obtainable with standards should be determined. A good way to reduce these sources of error is to add an accurately weighed amount of an internal standard to the mixture to be analyzed and compare the peak areas of the compounds to be measured against the peak area of the internal standard. The internal standard used in the present work was _myo_-inositol hexaacetate.

The response of the gas chromatography to each of the alditol acetates to be measured was calibrated by analyzing varying, but accurately weighed, amounts of each crystalline, authentic, alditol acetate with authentic, crystalline _myo_-inositol hexaacetate. The calibration factor for each alditol acetate was obtained by plotting the ratio of the area of each alditol acetate to the area of the standard (_myo_-inositol hexaacetate) as ordinate against the ratio of the weights of each as the abscissa. Straight lines passing through the origin resulted for arabinitol pentaacetate, xylitol pentaacetate, mannitol hexaacetate, galactitol hexaacetate, and glucitol hexaacetate. The slope of each of these lines is the "Instrument K Factor" and represents the overall response of the gas chromatographic system to each of the alditol acetates calibrated against the internal standard, *myo*-inositol hexaacetate. The "Instrument K Factors" were: arabinitol pentaacetate 0.96; xylitol pentaacetate 0.95; mannitol hexaacetate 0.96; galactitol hexaacetate 0.98; glucitol hexaacetate 0.92. These "Instrument K Factors" were used in subsequent calculations.

A sample of the holocellulose from Douglas-fir inner bark was carefully hydrolyzed to minimize acid degradation. The hydrolyzate so obtained was reduced by the addition of sodium borohydride and the excess sodium borohydride was decomposed by acetic acid. The solution was concentrated to a sirup. The sirup was dissolved in methanol and the methanol was reevaporated. This procedure was repeated a total of eight times. Albersheim, Nevins, English and Karr (32) showed that the boric acid level could be reduced to a convenient level by a number of additions and reevaporations of methanol. The boric acid was presumably converted to volatile methyl borate which was evaporated from the sirup. The removal of boric acid is important otherwise the acetylation reaction does not proceed. Acetylation and the gas-liquid chromatographic analysis was accomplished as outlined above.

The areas under the peaks were measured with a planimeter and were found to be (duplicate analyses): arabinitol pentaacetate, 13.2; xylitol pentaacetate, 39.7; mannitol hexaacetate, 62.0; galactitol hexaacetate, 15.4; glucitol hexaacetate, 386.0; *myo*-inositol hexaacetate, 232.2.

The following equation was used to determine the individual anhydromonosaccharide residue in the holocellulose fraction (31):

$$\text{\% anhydromonosaccharide residue} = \frac{C \times I \times F \times 100}{R \times S \times H \times K}$$

where

C = chromatographic area of the component alditol acetate peak,

R = chromatographic area of the *myo*-inositol hexaacetate peak,

I = weight of the *myo*-inositol originally added, in grams,

S = dry weight of the original holocellulose sample, in grams,

F = factor to convert weight monosaccharide to anhydromonosaccharide residue (0.88 pentose)(0.90 hexose),

H = hydrolysis survival factor (90),

K = "Instrument K Factor".

Using the area under the peak of glucitol hexaacetate as an example the calculations become:

$$\%\text{ anhydroglucose residue} = \frac{386.0 \times 100 \text{ mg} \times 0.90 \times 100}{232.2 \times 273.96 \text{ mg} \times 0.974 \times 0.92} = 61.1\%$$

In a similar way the percentages of anhydromonosaccharide residues in the Douglas-fir inner bark holocellulose were calculated (average of two determinations): anhydroarabinose residues, 2.6; anhydroxylose residues, 6.3; anhydromannose residues, 9.5; anhydrogalactose residues, 2.3; anhydroglucose residues, 61.1. The total yield of anhydromonosaccharides was 81.8%. This value is comparable to the values of 74.9%, 78.0% and 81.9% reported by Borchard and Piper (31) for the total carbohydrate content of chlorite holopulps and appears to be favorable for materials of this type.

The value of 81.8% carbohydrates plus the value of 7.2% lignin amounts for 89.0% of the solids in the holocellulose fraction. This total value is similar to the values usually reported for the solids content of materials of this type.

The study of the structure of bark hemicelluloses requires homogeneous polymers isolated in high yields with minimal modification. This is usually accomplished, as in the present work, by delignifying the bark and extracting the resulting holocellulose with aqueous alkali. Solubility differences can be exploited for the separation of individual hemicelluloses by judicious use of cations and concentrations. Thus, relatively dilute alkalis suffice to dissolve xylans and galactoglucomannans, but higher concentrations are required for the extraction of glucommannans.

The procedure used in the present work to separate the polysaccharides which comprise the holocellulose from Douglas-fir inner bark is outlined in Figure 7. The key feature was selective blocking of the dissolution of mannose-containing polysaccharides in the first extraction step. This was accomplished by impregnating the holocellulose with aqeuous barium hydroxide (55). The impregnated holocellulose was then contacted with 10.0% aqueous potassium hydroxide which was known to be a good solvent for xylan-rich polysaccharides. The extract was neutralized and methanol was added to 70.0% concentration. The resulting precipitate was labelled "Hemicellulose A." Hemicellulose A was recovered in 7.6% yield based on the starting holocellulose (Figure 7).

Residue A (Figure 7) remaining from the 10.0% potassium hydroxide extraction was expected to contain most of the mannose-containing hemicelluloses because of the impregnation with barium hydroxide (55). After removal of barium ions by dialysis, the residue was extracted with 1.0% aqueous sodium hydroxide which has been shown to be a good solvent for galactoglucomannan hemicelluloses (55).

The extract was neutralized and methanol was added to 70.0% concentration. The resulting precipitate was labelled "Hemicellulose B." Hemicellulose B was recovered in 1.7% yield based on the starting holocellulose (Figure 7).

The concentration of the sodium hydroxide extraction medium was increased to 15.0% sodium hydroxide because it has been shown (55) that polysaccharides rich in mannose residues become soluble in concentrated alkali solutions. The extract was neutralized and methanol was added to 70.0% concentration. The resulting precipitate was labelled "Hemicellulose C." Hemicellulose C was recovered in 2.9% yield based on the original holocellulose (Figure 7).

Residue C (Figure 7) was hydrolyzed by acid. Paper chromatography showed considerable amounts of mannose and galactose and traces of xylose and arabinose as well as glucose in the hydrolyzate. It was thought possible that increasing the sodium hydroxide concentration from 15.0% to 18.0% would extract more of the mannose- and galactose-containing polysaccharides and leave a relatively homogeneous glucan as a residue. However, the residue from the 18.0% sodium hydroxide still appeared to contain the same ratios of sugars. Therefore, further investigations were concentrated on the extract and residue from the 15.0% sodium hydroxide extraction (Figure 7) rather than those from the 18.0% sodium hydroxide extraction.

The minimum hydrolysis time for Hemicellulose A in acid to achieve the maximum yield of monosaccharide was determined to be 4 hours. A plot of glucose equivalents (%) versus reflux time in hours indicated complete hydrolysis after 4 hours and in most cases additional aliquots of the material were refluxed for 4.5 to 6 hours.

The maximum amount of glucose equivalents released was found to be 59.3%. However, the fraction contained 29.7% ash determined as the oxides. The major inorganic ion was considered to be Ba^{++} because of the previous impregnation with barium hydroxide. By calculation the percentage of barium ions in the fraction would be 26.6%. Therefore, the total solids accounted for in the fraction was 85.9%. Acid degradation of xylose containing polysaccharides is known to be extensive. In fact, the conversion of pentoses to furfural under conditions of acid reflux is the basis of the TAPPI standard method for the determination of pentosans in wood and pulp. The relatively low total recovery of materials in the Hemicellulose A fraction was, therefore, attributed to acid degradation.

A paper chromatogram of the acid hydrolyzate showed a very strong spot for xylose, weak spots for glucose, galactose, and arabinose and a trace spot for mannose. There was a slow moving spot which barely migrated from the origin. This spot was attributed to glucuronic acid.

The trace amount of mannose indicated that the impregnation of the holocellulose with barium hydroxide worked well in blocking the dissolution of mannan-containing hemicelluloses. These results are in agreement with those previously reported (55) for the separation of materials of this type.

The Hemicellulose A fraction was shown to be a "xylan" composed of the following ratio of sugar residues: xylose, 4.5; arabinose, 1.0; glucuronic acid, 1.0. The xylan possessed a specific rotation of -30.5. The intrinsic viscosity of the polysaccharide in molar deithylenediamine copper II reagent at 25° was 0.42 dl/g which corresponded to a degree of polymerization of 89. The molecular weight of the xylan as analyzed by end-group analysis was 1.8×10^4 which, in combination with gas-liquid chromatographic analysis, showed a degree of polymerization of 90.

The xylan was oxidized with periodate anion. It consumed 133.5 moles of periodate anion and released 22.5 moles of formic acid per mole of xylan. The oxidized xylan was hydrolyzed and analyzed by gas-liquid chromatography and showed the following ratio of sugar residues: xylose, 7.0; arabinose 1.0.

The above data were consistent with a polysaccharide xylan structure consisting of a backbone of 88 anhydro-D-xylopyranose units hooked to β-D-(1→4) plus a reducing and a non-reducing end group on the backbone. There were 20 anhydroglucuronic acid side chains and 20 anhydroarabinose side chains on these 90 units. At least ten of the anhydroarabinose units were in the form of monoarabinose side chains and up to ten were in the form of arabinobiose or longer side chains.

The xylan was reacted at 100° with aqueous sodium hydroxide solutions ranging in concentration from 0.001 N to 3.676 N. The rate of degradation did not change at alkali concentrations above 0.1 N in sodium hydroxide.

Alkaline degradation of the xylan in 0.1 N aqueous sodium hydroxide at 100° showed a rate constant for end-group peeling of $k_1 = k_2 = 5.33$ hour^{-1} and a termination rate constant of $k_3 = 0.66\ \text{hour}^{-1}$.

The peeling reaction stopped when 19.81% of the xylan had been degraded. This small amount of degradation reflected the relatively high termination rate constant compared to the peeling rate constant.

Hemicellulose B was hydrolyzed with sulfuric acid and the hydrolyzate qualitatively analyzed by paper chromatography. The chromatogram showed strong spots for glucose and mannose, weak spots for galactose and xylose, and a trace spot for arabinose.

The fraction still contained some xylose residues indicating that the extraction of the xylose-containing hemicelluloses was not complete by the extraction with 10.0% potassium hydroxide. Beélik, Conca, Hamilton and Partlow (55) also reported xylose residues in similar fractions isolated from western hemlock holocellulose and grand fir holocellulose. Hemicellulose B was considered to be a galactoglucomannan.

Hemicellulose C was hydrolyzed, neutralized, and analyzed by paper chromatography by the methods described for the holocellulose fraction. The chromatogram showed that the Hemicellulose C fraction was composed of a large amount of mannose residues, small amounts of glucose and galactose residues, and trace amounts of arabinose and xylose residues.

The results indicate that all of the xylan hemicelluloses had been extracted by the 10.0% potassium hydroxide and the 1.0% sodium hydroxide treatments. This is in agreement with the results reported by Beélik, Conca, Hamilton and Partlow (55) for hemicelluloses from softwoods.

The gas-liquid chromatographic spectrum of the alditol acetates prepared from the acid hydrolyzate of Hemicellulose C showed that the major peak present was from mannitol hexaacetate. The areas under the peaks of rhamnitol pentaacetate, arabinitol pentaacetate, and xylitol pentaacetate were too small to be measured. The areas under the remaining peaks as measured by a disc integrator were (average of triplicate reading): mannitol hexaacetate, 3407; galactitol hexaacetate, 315; glucitol hexaacetate, 240; myo-inositol hexaacetate, 11617. The percentages of anhydromonosaccharide residues were calculated similarily to those for the holocellulose fraction from the formula:

$$\% \text{ anhydromonosaccharide residue} = \frac{C \times I \times F \times 100}{R \times S \times H \times K}$$

The results were: anhydromannose residue, 85.7%; anhydrogalactose residue, 7.0%; anhydroglucose residue, 4.6%; total recovery, 97.3%.

The ratio of anhydrosugars in Hemicellulose C was therefore: anhydroglucose, 1.0; anhydrogalactose, 1.5; anhydromannose, 18.6. Therefore, the great majority of Hemicellulose C is composed of mannose-containing polysaccharides and is therefore termed a "mannan."

The specific rotation of the mannan was $[\alpha]_D^{23}$ - 40° (c 0.25, N sodium hydroxide). This is close to the rotations of mannans from other plant sources and supports the above conclusion that this fraction from Douglas-fir inner bark is primarily a mannan.

Methylation of a polysaccharide provides information about the positions of the glycosidic bonds which unite the anhydromonosaccharide residues in the polymer. The usual procedure is to form methyl ethers on all of the free hydroxyl groups. These ethers are resistant to the acid hydrolysis conditions ordinarily applied and so hydrolysis of the completely methylated polysaccharide frees only the hydroxyl groups involved in the glycosidic linkages. Location of the positions of the freed hydroxyl groups determines the point of attachment of the glycosidic bond in the original polymer.

It is important that all of the hydroxyl groups in the original polysaccharide be methylated. The methylation reaction is usually analyzed by infrared spectroscopy because the hydroxyl groups absorb readily and their disappearance as methylation progresses can be followed.

In the present work with the mannan from Douglas-fir inner bark, methylation proved difficult. However, after extended reaction time the infrared spectrum showed no absorption for hydroxyl groups in the usual regions of 3800 $\sim$ 3300 cm^{-1}. Therefore, methylation was considered complete.

Formic acid has been shown to be a good medium for the hydrolysis of mannans and was used in the present work. The initial formic acid treatment must be followed by an hydrolysis with dilute sulfuric acid to hydrolyze any formate esters which might have been formed.

Paper chromatography of the hydrolyzate sirup in two different solvent systems showed R_f and R_G values consistent with the presence of 2,3,6-tri-O-methyl-D-mannopyranose (very strong spot), 2,3,4,6-tetra-O-methyl-D-mannopyranose (trace spot) and 2.3-di-O-methyl-D-mannopyranose (trace spot). There were several unidentified spots which are possibly partially methylated sugars resulting from an incomplete methylation of the mannan as is evidenced by the presence of 2,3-di-O-methyl-D-mannopyranose. They were present in very trace amounts and could only be detected when the paper chromatogram was heavily loaded with hydrolyzate sirup. On the other hand, the spot for 2,3,6-tri-O-methyl-D-mannopyranose was very strong.

The crystallization of the above derivative of 2,3,6-tri-O-methyl-D-mannopyranose shows that the hydroxyl group on C4 of the mannopyranose compound was freed on hydrolysis of the methylated mannan. The isolation of the crystalline derivative supports the paper chromatographic evidence for the presence of 2,3,6-tri-O-methyl-D-mannopyranose in the hydrolyzate of the methylated mannan. Moreover the paper chromatograms show it to be not only the major spot but essentially the only spot.

Therefore, it is concluded the Hemicellulose C is primarily a mannose containing polysaccharide and that the anhydromannose residues are attached by β-D-(1→4)-glycosidic bonds. The β configuration is taken from the negative optical rotation (-40°).

A paper chromatogram of the acid hydrolyzate of Residue C showed a strong spot for glucose, medium spots for mannose and galactose, and trace spots for arabinose and xylose. Residue C therefore appeared to be a mixture of the hemicelluloses now known to be present in the holocellulose from Douglas-fir inner bark. While the extractions shown in Figure 7 tended to dissolve relatively pure hemicelluloses, the final residue was still a mixture.

Formic acid has been shown to completely remove hemicelluloses from an anhydroglucose rich material and leave a residue containing only anhydroglucose residues (70). The degradation of the residue in this procedure can be quite extensive and relatively short treatment of 4-hour duration was first attempted. However, a paper chromatogram of the hydrolyzate of the residue remaining after the 4-hour extraction showed a weak spot for mannose, and a trace spot for xylose and arabinose as well as the strong spot for glucose. Therefore, removal of the hemicelluloses was not complete.

Longer extraction time in the refluxing formic acid was attempted. The yield of residue was 36.0% and so degradation was quite extensive. However, a paper chromatogram of the acid hydrolyzate of the residue showed the presence of glucose only with no contamination from other sugars. Therefore, the other hemicellulose linkages had been hydrolyzed by the formic acid in preference to those joining the anhydroglucose residues. Therefore, a glucan was left as the residue.

Since the material under investigation was a glucan, the Somogyi copper reduction method with glucose as the standard sugar would give a reliable quantitative analysis of the amount of glucan in the sample. The amount of reflux time required for total hydrolysis was also determined by removing aliquots as the reaction progressed and determining the reducing power.

The hydrolysis was complete after 4 hours of reflux and the overall yield in glucose equivalents was 103.3%. Values of greater than 100% recovery occur sometimes in this type of procedure due to the accumulative sources of error in moisture content determinations, dilution measurements and the error of the Somogyi method. The large yield does show that the residue remaining after extracting Residue C (Figure 7) for 4 days with formic acid was a glucan.

The gas-liquid chromatographic spectrum of the alditol acetate prepared from the acid hydrolyzate of the glucan showed trace peaks for rhamnitol pentaacetate, arabinitol pentaacetate, and xylitol pentaacetate. The areas of these peaks were too small to be measured. Primarily the spectrum shows peaks for glucitol hexaacetate and the added myo-inositol hexaacetate. The areas under these peaks, as measured by a planimeter were (average of three readings): glucitol hexaacetate, 41; myo-inositol hexaacetate, 165. The percentage of anhydroglucose residues was calculated similarly to those for the holocellulose fraction from the formula:

$$\%\ \text{anhydroglucose residue} = \frac{C \times I \times F \times 100}{R \times S \times H \times K}$$

The results showed the percent of anhydroglucose residues to be 94.4%.

A definitive method of establishing linkages in a polysaccharide is by fragmentation analysis. If known dimers can be identified, then the linkage joining the two anhydromonosaccharide units is established. In the present investigation of the glucan, cellobiose octaacetate was isolated in crystalline form from the glucan. Thus the presence of β-D-(1→4) glycosidic bonds in the glucan was established. It is interesting to note, however that the time of acetolysis had to be shortened from two weeks, which is the usual reaction time for cellulose (70) to one week. No cellobiose octaacetate was obtained if the acetolysis of the glucan from Douglas-fir bark was allowed to react for two weeks. This would indicate that the chain length was short, because if the acetolysis proceeds too far on a glucan then glucose pentaacetate is produced rather than the dimer. Thus the acetolysis showed the presence of β-D-(1→4) glucosidic bonds, but indications were of a short chain.

The calculations of the viscosity data were made as follows:

$\eta = K\rho t$

$\eta_r = \eta/\eta_o$

$\eta_{sp} = (\eta-\eta_o)/\eta_o = \eta_r - 1$

$\eta_{sp/C}$ = reduced viscosity

$[\eta] = \lim_{C\to 0} (\eta_{sp}/C)$

where

η = viscosity in centipoise (cp)

η_o = viscosity in pure solvent

η_p = relative viscosity

$[\eta]$ = intrinsic viscosity in deciliters/gram (dl/g)

K = viscometer constant

ρ = density at 25.0°±0.01

t = time in seconds for solution to flow through the viscometer

c = concentration in g/dl

The plot of the reduced viscosities against the concentration showed a straight line as determined by linear regression analysis. The value for the intrinsic viscosity was obtained by extrapolating the plots to zero concentration. This was determined to be 0.43 dl/g.

The degree of polymerization (DP) of glucans from their intrinsic viscosities in cupriethylenediamine copper II solution can be calculated using the equation

$$[\eta] = 0.0098\ DP^{0.9}$$

Using the above relationship and a value of 0.43 dl/g for the intrinsic viscosity, a DP of 67 is obtained for the glucan. This value shows quite a short chain length for the glucan. This however, is consistent with the difficulties encountered in isolating crystalline cellobiose octaacetate from acetolysis of the glucan because the chain length appeared so short that the acetolysis was going all the way to glucose pentaacetate rather than stopping at cellobiose octaacetate. It is quite likely that the rigorous extraction with formic acid for four days cleaved the glucosidic bonds and resulted in short chains.

The glucan was methylated to substitute all of the hydroxyl groups prior to hydrolysis. The infrared spectra of the methylated glucan showed the disappearance of the hydroxyl absorption in the 3800 - 3300 cm^{-1} range indicating that all of the hydroxyl groups had been methylated.

The methylated glucan was hydrolyzed with sulfuric acid in the same way that the glucan had been hydrolyzed. Paper chromatography in two different solvent systems showed R_f and R_G values consistent with the presence of 2,3,4,6-tetra-O-methyl-D-glucopyranose (trace spot), and 2,3,6-tri-O-methyl-D-glucopyranose (very strong spot). The unidentified spots are possibly partially methylated sugars resulting from slightly incomplete methylation of the glucan. These materials were present in only trace amounts and could be detected only when the chromatograms were heavily loaded with hydrolyzate sirup. On the other hand the spot for 2,3,6-tri-O-methyl-D-glucopyranose was very pronounced.

The isolation of crystalline cellobiose octoaacetate from acetolysis of the glucan proved the presence of β-D-(1→4) linkages and hence established the presence of 2,3,6-tri-O-methyl-D-glucopyranose in the hydrolyzate from the methylated glucan. Thus the identification of 2,3,4,6-tetra-O-methyl-D-glucopyranose by comparing relative spot movements on paper chromatograms was placed on firm ground. This material came from the non-reducing end group of the glucan.

The above data on the glucan is consistent with the presence of a linear polysaccharide of 65 repeating anhydroglucopyranose units attached by β-D-(1→4) glucosidic bonds plus a reducing and non-reducing end group.

DOUGLAS-FIR BARK WAX

I. Historical Review

Kurth and Kiefer (71) found that the chemical components of the whole bark could be partially separated by extraction with various solvents. Table 6 indicates the nature and yields of the Douglas-fir extractives which they removed by successive extraction with the solvents shown.

Kurth, in a separate publication (72), reported on the chemical composition of the "waxes" in Douglas-fir bark. The n-hexane-soluble wax was saponified by refluxing a mixture of 20 g of the wax, 20 g of potassium hydroxide and 300 ml of 70% ethanol for three hours. Following this, 100 ml of water was added, the alcohol was removed by evaporation, and the residue extracted with n-hexane in a separatory funnel to remove unsaponifiable compounds. The unsaponifiable matter was dried and recrystallized from acetone. A white crystalline solid was obtained which was identified as lignoceryl alcohol, also named tetracosanol-1, a straight-chained, saturated alcohol, $C_{24}H_{50}OH$. The yield was 19.5% of the "wax." The filtrate from the lignoceryl alcohol crystallization gave a positive Liebermann-Burchard test for sterols (73, p. 80; 74, p. 100) and a precipitate with digitonin. After removal of the acetone, the residue was crystallized as white needles from dilute alcohol. The material was identified as "phytosterols." The yield was 0.3% of the "wax." "Phytosterols" are now known to be mixtures of closely related sterols of which the most common one in bark is β-sitosterol.

The alkaline solution from the separation of the unsaponifiable matter was acidified with sulfuric acid and extracted with n-hexane in a separatory funnel. The n-hexane solution was washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness. The residue was recrystallized from acetone and then from n-hexane. The white crystals were identified as lignoceric acid, also named tetracosanoic acid, a straight-chained, saturated acid, $C_{24}H_{48}O_2$. The yield was 60.5% of the "wax." The small amount of yellow solid residue left in the filtrate from this crystallization was oxidized with cold potassium permanganate to dihydroxystearic acid; m.p. 129-130°. This indicated the presence of oleic acid in the original wax.

A brown resin remained suspended in the aqueous liquor after the n-hexane extraction of the lignoceric acid. The resin was extracted with diethyl ether. Evaporation of the ether left a brown residue which crystallized from benzene as fine crystals. Recrystallization from dilute ethanol gave yellow prisms. These crystals were identified as ferulic acid (4-hydroxy-3-methoxycinnamic acid). The yield was 22% of the original wax.

The fraction of the bark which was n-hexane insoluble but benzene soluble (benzene-soluble "wax") was reported by Kurth and co-workers (75, 76) to possess a more complicated composition than the n-hexane-soluble "wax." It had a melting point of 60° to 63°, and Kurth tentatively isolated, after saponification, a fatty acid mixture in about 25% yield, a dark-colored phlobaphene in 24% yield, a dark colored diethyl ether-soluble acid fraction in 26% yield, unsaponifiable matter in 5% yield and glycerol.

The cork fraction of Douglas-fir bark was studied by Hergert and Kurth (76). The extractives were separated according to their solubilities in n-hexane, benzene, diethyl ether, ethanol and hot water. The n-hexane and benzene fractions were "waxes." The yield of n-hexane-soluble "wax" was 5.78% and of benzene-soluble "wax" was 1.75% based on an average of nine trees sampled. The n-hexane-soluble "wax" was found to contain lignoceric acid, 49.3%; lignoceryl alcohol, 27.5%; ferulic acid, 9.8%; "phytosterol", 0.6%; and n-hexane-insoluble, benzene-soluble acidic material, 8.1%; a benzene-insoluble phenolic material, 3.6%. The n-hexane-insoluble but benzene-soluble "wax" proved to have a complicated composition. The authors did not locate the position of the hydroxyl group in the hydroxypalmitic acid, $C_{16}H_{32}O_3$, but reported that it was not terminal.

It is interesting to note that dihydroquercetin was the extractive present in greatest amount from the cork. Although not extracted with n-hexane or benzene, and not considered a part of the "wax-portion," Douglas-fir bark represents a major source of this compound. It is easily obtained from the "wax"-free (benzene extracted) bark or cork by extraction with diethyl ether. Upon evaporation of the diethyl ether it can be recrystallized from hot water as long white needles, m.p. 241-248 (71). The quantity of dihydroquercetin in cork varies from 5% in samples of cork from second-growth trees to 23% in cork from mature trees (76). This indicates the desirability of separating the cork from the other bark components when the extraction of dihydroquercetin is intended.

In 1967 Kurth reported more recent work on the n-hexane-insoluble, benzene-soluble "waxes" of Douglas-fir bark and cork (75). In addition to the compounds previously mentioned, he found an hydroxybehenic acid $C_{22}H_{44}O_3$, and an hydroxyarachidic acid (hydroxyeicosanoic acid), $C_{20}H_{40}O_3$.

The hydroxybehenic acid was crystalline but gave a range of melting points depending upon how it was recrystallized. Kurth comments that this is characteristic of those hydroxy-fatty acids that form lactones and lactides. He did not report the position of the hydroxyl group. Likewise, he did not report the exact position of the hydroxyl group in the hydroxyarachidic acid. He also mentions the presence of a dicarboxylic acid of tentative formula $C_{20}H_{38}O_4$ but did not report further characterization.

The chemical composition of the bast fibers of Douglas-fir bark were also studied by Kiefer and Kurth (77). Table 6 shows the yields of extractives resulting from successive extractions with the solvents shown. They indicated that the bast fibers did not contain as much extractive material, particularly dihydroquercetin and "wax," as did the cork fraction of Douglas-fir bark. Further separation of the extractives into their individual components was not reported.

II. Experimental: n-Hexane-Soluble Wax

A. Collection of Bark Samples

Bark samples used in this study were collected from indigenous sources of Douglas-fir. They consisted of bark from trees of various ages and diameters. The specimens were stripped from the trees, sealed in polyethylene bags, and brought to the Forest Research Laboratory, Oregon State University.

B. Sample Preparation and n-Hexane Extraction

Bark chips were ground to pass a screen of ten meshes to the inch (The W. S. Tyler Company, Cleveland, Ohio). Since it was not necessary to dry the bark before extraction (57), the ground bark was packed into a Soxhlet thimble, placed in a Soxhlet extractor and extracted for 48 hours with n-hexane. The solvent was evaporated under aspirator vacuum on a rotary evaporator (Buchi, Rotavapor, Switzerland). The residual "wax-like" solid was transferred to a sample jar with a minimum of n-hexane solvent. The excess solvent was evaporated by passage of a stream of nitrogen. The ultraviolet absorption spectrum of the extracted solids was measured in a chloroform-n-hexane (3:1 v/v) solution.

C. Separation of the n-Hexane-Soluble Components

1. Separation by Column Chromatography

Column chromatrography was used as a first step in separating the n-hexane-soluble materials. A typical separation is described below. Silica Gel G was packed uniformly into a glass column two feet in length and one inch in diameter. A portion (2.00 g) of the n-hexane-soluble fraction was dissolved in chloroform (15.0 ml) and added to the top of the Silica Gel G column. Chloroform-n-hexane (3:1 v/v) was used as the developing solvent. A series of bands were located by use of ultraviolet light.

The fastest moving two bands (blue and light blue bands) were investigated by H. Aft (personal communication). The present investigation was concerned with the next two fastest moving bands, the light bluish-green band and the bright-blue band (designated in combination as the "yellow band"). To collect these bands the fastest moving zones were first washed from the column and stored. Becuase of the difficulty of separating the next two bands, they were washed from the column and collected as a mixture. The mixture was added to the top of a second Silica Gel G column and developed with the same solvent system. Then the bright-blue band (fastest moving) approached the sand in the bottom of the column, fractions of effluent (5.0 ml) were collected. These fractions were tested by thin-layer chromatography. The fractions were tested in two different solvent systems: diethyl ether-n-hexane (1:4 v/v) and chloroform-carbon tetrachloride (6:1 v/v). The chromatograms showed several spots for each fraction, indicating that the column chromatographic separation was incomplete.

However, as a source of starting meterial for further separations, the "yellow band" was collected from several Silica Gel G columns.

The Liebermann-Burchard test (59, p. 70; 60, p. 100) was employed to determine if the material contained unsaturated sterols. A sample of the "yellow band" from the column chromatographic separation was dissolved in chloroform. An aliquot (1.0 ml) of the solution was added to a test tube and dried. Acetic anhydride (2.0 ml) was added followed by the dropwise addition of concentrated sulfuric acid. A dark green color change indicated a positive test.

2. Separation by Thin-Layer Chromatography

The "yellow band" from the column chromatographic separation was subjected to thin-layer chromatography in several solvent systems in an effort to more completely resolve the mixture.

Solutions of the samples to be chromatographed were applied to the thin-layer plates with capillary tubes in such a way that the surfaces of the plates were disturbed as little as possible. The chromatography tanks used for irrigating the thin-layer plates were saturated with vapors from the developing solvent at least two hours before the plates were placed in the tanks.

Aliquots of the "yellow band" in chloroform-_n_-hexane (3:1 v/v) solution were added to the thin-layer plates and developed with the following solvent systems (all hexane solvents were _n_-hexane):

1. Chloroform-carbon tetrachloride (6:1 v/v)
2. Chloroform-carbon tetrachloride (1:1 v/v)
3. Chloroform-carbon tetrachloride (1:4 v/v)
4. Chloroform-hexane (4:1 v/v)
5. Benzene-hexane (2:1 v/v)
6. Diethyl ether-hexane (3:1 v/v)
7. Diethyl ether-hexane (1:4 v/v)
8. Chloroform-benzene (6:1 v/v)
9. Chloroform-benzene (1:1 v/v)
10. Chloroform-benzene (1:4 v/v)
11. Benzene-methanol-acetic acid (45:8:4 v/v/v)
12. Hexane-diethyl ether-acetic acid (70:30:1 v/v/v)
13. Hexane-diethyl ether-acetic acid (85:15:1 v/v/v)

14. Chloroform-ethyl acetate-formic acid (5:4:1 v/v/v)

Some thin-layer plates were developed two dimmensionally in the following solvent systems:

n-hexane-diethyl ether-acetic acid (70:30:1 v/v/v) in one direction followed by chloroform in the second direction; diethyl ether-n-hexane (1:4 v/v) in one direction followed by chloroform-carbon tetrachloride (6:1 v/v) in the second direction.

After development, the thin-layer plates were dried at room temperature. Those plates which were impregnated with silver nitrate were dried in the dark. The following methods were used to examine the developed plates.

1. Ultraviolet light
2. Ultraviolet light with the plate in ammonia vapors
3. Sprayed with 30% aqueous sulfuric acid, heated for 5 to 10 min at 105° in an oven and finally viewed under ultraviolet light.
4. Exposure to iodine vapors.
5. Sprayed with bromothymol blue solution (50 mg bromothymol blue, 1.25 g boric acid, 8 ml 1 N sodium hydroxide and 112 ml water, or 40 mg bromothymol blue and 100 ml 0.01 N sodium hydroxide)
6. Sprayed with p-nitroaniline solution ((a)p-nitroaniline, 0.3% in 8% (w/v) HCl (25 ml) and $NaNO_2$ (5% w/v)(1.5 ml) mixed immediately before spraying. (b) Na_2CO_3 (20% w/v)). The two solutions were used successively.

Authentic β-sitosterol (Aldrich Chemical Co., Inc., Milwaukee, Wisconsin) was chromatographed by one-dimensional thin-layer chromatography simultaneously with the "yellow band" in each of the 14 solvent systems listed above. The spots were located by exposure to iodine vapor. The separation by every solvent system showed a compound in the "yellow band" which migrated the same distance as the authentic β-sitosterol.

3. Separation by Gas-Liquid Chromatography

Gas-liquid chromatography was used to test the purity of the samples separated by column chromatography and thin-layer chromatography and to collect pure samples. A Hewlett-Packard 5751B Research Chromatograph (Hewlett-Packard Company, Palo Alto, California) were used. Both instruments were equipped with flame ionization detectors and used helium as the carrier gas. Stainless steel columns (1/8 in O.D.) were filled with the packing material of choice. A glass wool plug was inserted in the end of the column and the material was packed to a uniform tightness by vibrating the column with a small vibrating tool. Four different column packings were used in an attempt to find a column packing which would give good resolution. Details of the four columns are listed in Table 7.

The columns were put on the instrument for conditioning for 12 to 18 hr at a temperature of 10° above the maximum temperature used for resolution. Various temperatures, flow rates, sensitivities and chart speeds were tested. Both temperature programming and isothermal methods were used.

Most of the samples were directly injected into the gas chromatograph in n-hexane solution. However, because some of the compounds were thought to contain hydroxyl groups the trimethylsilyl ethers of several of the fractions were prepared in an attempt to improve resolution. These trimethylsilyl ethers were made by reacting dry samples for 5 to 10 min at room temperature with hexamethyldisilazane and trimethylchlorosilane in pyridine (2:1:10 v/v/v). The pyridine solution was injected directly into the gas chromatograph.

The "yellow band" from column chromatography was investigated. Four different columns were used (Table 7). The experimental conditions are shown in Table 8.

Two bands from the thin-layer chromatographic separation [developer, diethyl ether-n-hexane (1:4 v/v)] of the "yellow band" were tested for purity by gas-liquid chromatography. The two bands were readily observed on the thin-layer plates by ultraviolet light. The faster moving band showed a yellow-blue color and the slower moving band showed a bright-blue color. These two bands were separately scraped from the thin-layer plates, and the organic compounds extracted from the solid Silica Gel G with n-hexane. The compounds were separated by gas-liquid chromatography on the following column packings: SE-32; US W-98; OV-17 (Table 10). Both silylated and non-silylated samples were injected under the general conditions outlined in Table 8.

An authentic sample of β-sitosterol was subjected to gas-liquid chromatography using the columns and the conditions shown in Table 8. The retention times were compared with those for the peaks in the unknown.

A "peak enhancement" study was conducted by adding a small quantity of authentic β-sitosterol to the n-hexane solution of the "yellow band" and injecting the solution into the gas chromatograph under the conditions outlined in Table 8.

A known sample of stigmasterol (Aldrich Chemical Co., Milwaukee, Wisconsin) was chromatographed on SE-52 and OV-17 columns under the conditions outlined in Table 8.

4. Identification by Gas-Liquid Chromatography Combined With Rapid-Scan Mass Spectrometry

Gas-liquid chromatography in combination with mass spectrometry was used to separate and identify a range of compounds present in the n-hexane-soluble fraction of Douglas-fir bark. The following operating conditions were used:

Gas-Liquid Chromatography

Instrument	F & M 810 (Hewlett-Packard)
Detector	Hydrogen flame ionization
Detector temperature	248°
Injection sample	4 μl of the silylated mixture
Injection port temperature	270°
Column	UC W-98
Column temperature	Isothermal at 265°
Carrier gas flow rate	30 ml/min of helium

Mass Spectrometry

Filament current	70 eV source, 40 μA
Electron voltage	20 eV and 70 eV
Analyzer pressure	1 x 10^{-6} mm Hg
Multiplier voltage	3.00 KV
Scanning speed	6.5 seconds from m/e 24 to 500

The mass spectrometer used was an Atlas CH-4, Nier-type (nine inch, 60 degree sector) single-focusing instrument. The trimethylsilyl ethers of the fractions of interest were separated by gas-liquid chromatography and a portion of the effluent was passed directly into the dual ion source of the mass spectrometer. The gas-liquid chromatograph was fitted with a 5:1 (EC-1 valve:flame) splitter. The mass spectrometer was equipped with an EC-1 throttle valve which was adjusted to permit approximately 10% of the remaining column effluent to enter the ionization chamber, the remaining effluent being vented into the air through a heated tube. The effluent was further split in the ion source with 50% going to the 70 eV source. The 20 eV source operated at less than the ionization potential of the carrier gas (helium), but above that of organic compounds, and was therefore used as a continuous total ionization readout without any contribution from ionized helium. The 70 eV source provided the ionization used to obtain the mass spectra. The spectra were recorded on a Honeywell 1508 oscillograph.

III. Experimental: Benzene-Soluble Wax (78, 79)

A. Bark Collection and Solvent Extraction

Whole bark was stripped from the bottom end of a freshly-felled, 58 year old Douglas-fir at Black Rock, Oregon. The bark was air-dried (moisture content 9.3%), ground to pass a 1/2-inch screen, and a portion (768.2 g dry cut) was packed into a stainless-steel mesh basket and extracted in a large Soxhlet extractor with n-hexane (8.0 liters) for 52 hr. The extract was filtered while hot through glass wool and the n-hexane evaporated on a rotary evaporator at 30° or less leaving a cream-colored, "wax-like" solid; yield, 30.0 g. This n-hexane "wax" was stored for future investigations.

The bark residue from the n-hexane extraction was air-dried, re-packed into the stainless-steel mesh basket and extracted with benzene (8.0 liters) in the Soxhlet extractor for 52 hr. The extract was filtered, and concentrated as above. The final traces of benzene were removed by freeze-drying, leaving a light-brown, "wax-like" solid; yield 20.0 g.

B. Saponification and Separation of Acids and Neutrals

An aliquot (10.0 g) of the benzene "wax" was saponified by gently refluxing for 3.75 hr in 10% NaOH (250.0 ml). The mixture was cooled in an ice bath and acidified with glacial acetic acid resulting in a precipitate. The organic compounds were recovered in two ways. In the first procedure the mixture was extracted with benzene without first collecting the precipitate. In the second procedure the precipitate was recovered on a filter, and then only the precipitate was extracted with benzene.

The benzene solution, containing both acids and neutrals, from both procedures was extracted in a beaker with 5% NaOH with warming and stirring. The aqueous layer was recovered and washed several times with fresh benzene. The benzene solution including washings, containing the neutrals, were washed first with dilute HOAc, then with water and dried with anhydrous Na_2SO_4.

The aqueous 5% NaOH fraction containing the acids was cooled in an ice bath, acidified with glacial acetic acid and extracted with benzene. A little diethyl ether was added to the benzene to break emulsions. The benzene solution was dried over anhydrous Na_2SO_4 and the solvent removed on a rotary evaporator at 30° or less leaving a tan-colored solid.

C. Preparation of Methyl Esters

The methanol used was reagent grade which had been doubly distilled through a short Vigreax column and checked by GLC with SE-30 as stationary phase.

The acids isolated above were dissolved in MeOH (400.0 ml) containing 5-6% gaseous HCl by weight. The brown solution was refluxed for 2-4 hr, cooled under tap water, concentrated to approximately 20 ml on a rotary evaporator and diluted with water (50-55 ml), resulting in a white precipitate. The mixture was extracted 3 times with 100 ml portions of doubly distilled n-hexane. The n-hexane extract was washed with 1% $NaHCO_3$, with water, and then dried with anhydrous Na_2SO_4. The n-hexane extract was removed on a rotary evaporator leaving a tan-colored solid.

D. Thin-Layer Chromatography (TLC)

The methyl esters isolated above were dissolved in benzene, diluted to 25 ml and aliquots were subjected to TLC on layers of pre-washed Silica Gel G using n-hexane-diethyl ether (70:30 v/v) as solvent and a spray reagent of

0.2% 2',7'-dichloroFluorescein as indicator. Authentic standard methyl esters were developed simultaneously with the unknown compounds.

Three major spots or "families" were evident. Spot No. 1 ($R_f0.74$) migrated essentially the same distance as authentic methyl lignocerate ($R_f0.76$) indicating that it was composed of non-oxygenated fatty acid methyl esters. Spot No. 2 ($R_f0.54$) migrated similarily to authentic dimethyloctadecanedioate ($R_f0.54$) indicating that it was composed of dicarboxylic fatty acid dimethyl esters.

Spot No. 3 ($R_f0.17$) was not readily identified by comparison with known standards but TLC and gas-liquid chromatographic (GLC) data, as well as properties of a derivative, leads to the belief that it contains hydroxy-acids.

To isolate the methyl ester "families" for further resolution by gas-liquid chromatography the methyl ester solution was applied as closely spaced spots to each of five TLC plates and developed as above. A narrow band on each plate edge was sprayed with 2',7'-dichloro-Fluorescein, and the whole plate briefly exposed to iodine vapors. As soon as the iodine color disappeared the spots were scraped from the plates and the organic compounds eluted by repeated washings with benzene. The eluents were concentrated to 1 ml or less for GLC.

E. TLC of the Neutral Fraction

The neutral fraction of benzene "wax" was subjected to TLC under the same condition as described above. No major spots resulted, spot A ($R_f0.30$) and spot B ($R_f0.07$). Spot B migrated the same distance as authentic β-sitosterol. In addition, the reaction of the following sprays used for the detection of steroids were noted: 85% phosphoric acid - H_2O (1:1 v/v); Liebermann-Burchard Reagent; Stahl $SbCl_3$-HOAc (1:1 v/v).

F. GLC of the Fatty Acid Methyl Esters

All GLC was performed on a Hewlett-Packard Model 5751B equipped with dual flame ionization detectors, a Model 17503A input module, a Model 7127A strip chart recorder, and a Series 300 DISK Integrator.

Column packings were prepared by coating Gas-Chrom Q (100/120 mesh) with the desired liquid phase and calculating the loading (w/w) by the filtrate measurement technique. All columns were made from 1/8 in O.D. stainless steel tubing. Specific columns used were: (a) 5 ft, 4.75% Silicone SE-30; (b) 2.5 ft, 8.25% Apiezon-L; (c) 3 ft, 7.4% Reoplex-400; (d) 6 ft, 11.1% EGGS-X. Various conditions and temperatures were used as specified in the results. Carrier gas (helium) flow rates were approximately 30 ml/min.

The methyl esters were injected into the GC in n-hexane or benzene solutions. Identification of GLC peaks were made by comparing retention times of the "unknown" peaks with a standard plot of chain-length vs. log retention time prepared from authentic standard compounds. Standard compounds were also added to the unknown samples for "peak enhancement" studies.

Peaks due to unsaturated compounds were located by comparing GLC's of bromine-treated samples with untreated samples.

GLC of standard mixtures of saturated and unsaturated methyl esters and of saturated diesters showed that peak areas very closely reflected the weight composition ratios of each series. In this way the ratios of the compounds of the two spots from TLC were calculated by comparing peak areas.

G. GLC Combined with Rapid-Scan Mass Spectrometry

Mass spectra (MS) of the compounds of Spot No. 2 (TLC) were obtained by tanden GLC-MS. GLC was performed on an F&M 810 (Hewlett-Packard) with the SE-30 solumn under conditions close to those already described. One-fifth of the column effluent was fed to the GLC flame ionization detector, while the remainder was passed to the heated EC-1 inlet of an Atlas MAT CH-4

Nier type mass spectrometer. The 20eV source provided constant monitoring of the organic compounds reaching the MS, while the 70eV source provided ionization for the spectra obtained by rapid magnetic scan.

H. Identification of the Alcohols

Identification of the alcohols of Spot A (section III-E) was made by co-GLC of the free alcohols (4.75% SE-30 and 7% Carbowax 20M) with authentic compounds, and by co-GLC (SE-30) of the acetates (Ac_2O in pyridine with toluenesulfonic acid), methyl ethers (CH_3I and Ag_2O), and TMSi ethers (HMDS and TMcS in pyridine) with the respective derivatives made from authentic compounds.

The C_{22} and C_{24} alcohols from benzene wax were collected from a preparative SE-30 solumn and identity was further verified by IR and m.m.p.

1. 1-Docosanol

1-Docosanol from benzene wax had m.p. 69.5-71°, which was not depressed by mixture with approximately equal amounts of authentic behenyl alcohol; IR (KBr), cm^{-1}: 3340 (broad) with shoulder ca 3230 (-OH stretch, H-bonded), 2910 and 2840 (alkyl), 1470 and 1460 (alkyl), 1056 (primary -OH), 723 and 712 ($-(CH_2)_n-$), and identical to spectrum of authentic behenyl alcohol. Acetate IR (KBr), cm^{-1}: 2915 and 2850 (alkyl stretch), 1745 (ester carbonyl), 1465 (alkyl), 1365 ($-CH_3$), 1235 (C-O-C for acetate), 1040 (C-O-C), 715 ($-(CH_2)_n-$), and identical to spectrum of acetate prepared from authentic alcohol.

2. 1-Tetracosanol

1-Tetracosanol from benzene wax had m.p. 76.0-76.5°, identical to that of authentic lignoceryl alcohol; IR (KBr), cm^{-1}: 3330 (broad) with shoulder ca 3230 (-OH stretch, H-bonded), 2910 and 2840 (alkyl), 1470 and 1460 (alkyl), 1056 (primary -OH), 722 and 712 ($-(CH_2)_n-$), and identical to a spectrum of authentic lignoceryl alcohol.

I. Identification of the Hydroxy Acids

Hydroxy methyl esters and the methoxy methyl esters were collected from a preparative SE-30 column for further analysis.

1. Methyl 16-hydroxyhexadecanoate

Methyl 16-hydroxyhexadecanoate from benzene wax had m.p. 54-55° (benzene) (lit. 54.5-55.5°); IR (KBr), cm^{-1}: 3400 with shoulder *ca* 3320 (hydroxyl), 2920 and 2850 (alkyl), 1740 (ester carbonyl), 1470 and 1463 (alkyl), 1435 ($-CH_3$, methyl ester), 1362, 1340, series of small bands 1190-1300 ($-CH_2-$ wag and twist), 1160 (methyl ester C-O-C), 1060 and 1045 (C-O), 880 ($-CH_2-COOCH_3$), 730 and 720 ($-(CH_2)_n-$rock). (Found: C, 71.5; H, 12.0. Calc. for $C_{17}H_{34}O_3$: C, 71.3; H, 12.0)

Treatment with HI and red P followed by Zn and HCl, and methylation (MeOH - HCl) gave one product whose retention time (SE-30 and EGSS-X) was the same as that of authentic methyl hexadecanoate, and the MS of the product showed the ion fragments expected for methyl hexadecanoate.

Oxidation (CrO_3 in HOAc) gave a product whose methyl ester had the same TLC R_f, the same R_t (SE-30 and EGSS-X), and the same MS as authentic dimethyl hexadecanedioate.

Methyl 16-methoxyhexadecanoate gave IR (KBr)cm^{-1}: 2995 (weak), 2915, 2855 and 2815 (weak) (alkane), 1740 (ester carbonyl), 1475 (alkyl), 1440, 1380, 8 sharp bands of varying intensity 1345-1203, 1170 (methyl ester C-O-C), 1110 implies ether C-O-C), 940, 880, and 715 ($(CH_2)_n$). GLC-MS, m/e: 300 (M^+), 285 ($M-CH_3$), 270 (M-30), 269 (M-31), 268 (M-32), 253 (M-47), 241 (M-59), 236 (M-64), 208 (M-92); also the following fragments (which were common to all homologs): 143, 129, 115, 101, 87, 74 (methyl ester), 55, and 45 ($CH_3OCH_2^+$, base peak).

2. Methyl 18-hydroxyoctadecanoate

White crystals from hexane had m.p. 62-63° (lit. 62.5°). IR (KBr) was identical to a published spectrum: 3390 cm^{-1} with shoulder at ca 3330 (-OH), 2910 and 2840 (alkane), 1740 (ester carbonyl), 1475 and 1468 (alkane), 1440 ($-CH_3$, methyl ester), 1370 and 1355, 1330. Series of small bands 1180-1310 ($-CH_2$ - wag and twist), 1170 (methyl ester C-O-C), 1055 (C-O), 883 ($CH_2 - COOCH_3$), 730 and 720 ($-(CH_2)_n$ - rock). The MS of the methoxy methyl ester was identical to a published spectrum of methyl 18-methoxyoctadecanoate: m/e 328, 313, 298, 297, 296, 281, 269, 264, 236 (assignments as for C_{16} homolog); m/e 143 and lower was identical to that for the C_{16} homolog.

3. Methyl 20-hydroxyeicosanoate

White crystals from hexane had m.p. 69.0-69.5° (lit. 68-68.5°); IR (KBr), cm^{-1}: ca 3330 (broad, -OH, H-bonded), 2910 and 2840 (alkyl), 1740 (ester carbonyl), 1475 and 1465 (alkyl), 1438 ($-CH_3$, methyl ester), 1385, series of 8 small bands 1310-1185 ($-CH_2$ - wag and twist), 1175 (methyl ester C-O-C), 1055 (C-O), 880 ($-CH_2-COOCH_3$), 728 and 715 ($-(CH_2)_n-$).

Reduction of the hydroxyl group gave a product whose methyl ester had the same R_t as authentic methyl eicosanoate (SE-30, EGSS-X); the MS contained ion fragments expected for methyl eicosanoate. Oxidation (CrO_3 in HOAc) followed by methylation gave a product whose TLC R_f, R_t (SE-30, EGSS-X), and MS were identical to those of dimethyl eicosandeioate isolated previously from benzene wax.

The methoxy methyl ester gave a MS similar to that of the lower homologs: m/e 356, 341, 326, 325, 324, 309, 297, 292, 264 (assignments as for C_{16} homolog); m/e 143 and lower was identical to lower homologs.

4. Methyl 22-hydroxydocosanoate

White crystals from hexane had m.p. 74.5-75.5° (lit. 73.5-74.5°). IR (KBr) was similar to that of the lower homologs; there was a series of 9 small bands 1315-1185 cm^{-1} ($-CH_2$ - wag and twist).

Reduction of the hydroxyl group gave a product whose methyl ester had the same R_t (SE-30, EGSS-X) as authentic methyl docosanoate; the MS contained ion fragments expected for methyl docosanoate. Oxidation (CrO_3 in HOAc) and methylation gave a product whose TLC R_f, R_t (SE-30. EGSS-X) and MS were identical to those of authentic dimethyl docosanedioate.

The methoxy methyl ester gave a MS similar to those for the lower homologs: m/e 384, 369, 354, 353, 352, 337, 325, 320, 292; fragmentation m/e 143 and below was identical to that for lower homologs.

5. Methyl 24-hydroxytetracosanoate

White crystals from hexane had m.p. 80-81° (lit. 80-81°). IR (KBr) was similar to that of the lower homologs; there was a series of 10 small bands 1310-1180 cm^{-1} ($-CH_2$ - wag and twist).

IV. Results and Discussion

Since a reasonably representative sample of Douglas-fir bark was desired, small amounts were taken from a number of trees in the area of Corvallis, Oregon. Because of the number of trees sampled and because these samples were thoroughly mixed into a larger collection, no effort was made to ascertain the diameter or age of the trees.

The n-hexane extraction closely followed the method of Kurth and Kiefer (71). These authors reported the n-hexane-soluble material to be a light colored "wax," whereas other solvents such as benzene extracted dark colored materials. The light color indicated a less complex fraction which was more desirable for an initial study.

Column chromatography on Silica Gel G was used to provide a coarse separation of the n-hexane-soluble compounds. A number of distinct bands were evident as the column developed.

The following bands labelled "light bluish green" and "bright blue" (designated in combination as the "yellow band" was eluted from the column and tested for purity by thin-layer chromatography. The better resolving power of the thin-layer technique showed that the "yellow band" was a mixture of several chemical compounds. Some thin-layer plates showed as many as 11 distinct spots plus a sizable spot which remained at the origin. The spot at the origin contained an undetermined number of compounds.

A solution of the "yellow band" showed a positive Lieberman-Burchard test (73, 74) indicative of the presence of sterols.

A quantity of the "yellow band" was accumulated from several columns and the mixture was further separated by thin-layer and gas-liquid chromatography.

The extensive thin-layer chromatographic study was conducted to find out how many compounds were in the "yellow band" and to find a solvent system which gave good separation which could be used as a method to collect pure compounds.

The 14 solvent systems employed are widely used for the separation of lipids as well as for sterols and terpenes. The solvent systems composed of chloroform-carbon tetrachloride (6:1 v/v), diethyl ether-n-hexane (1:4 v/v), n-hexane-diethyl ether-acetic acid (70:30:1 v/v/v) were the best. Of these three, chloroform-carbon tetrachloride (6:1 v/v) produced more spots than the other two, but each spot had considerable tailing. The diethyl ether-n-hexane (1:4 v/v) system gave very good separation and although the chromatogram showed fewer spots than the solvent system of chloroform-carbon tetrachloride (6:1 v/v) the amount of tailing was reduced. The addition of acetic acid to the ether and n-hexane solvent systems [n-hexane-diethyl ether-acetic acid (70:30:1 v/v/v), and n-nexane-diethyl ether-acetic acid

(65:15:1 v/v/v)] did not show improvement. Solvent systems with higher polarity such as benzene-methanol-acetic acid (45:8:4 v/v/v) and chloroform-diethyl ether-formic acid (5:4:1 v/v/v) showed poor separation and fewer spots could be observed than with the less polar solvent systems.

Exposure of the thin-layer plates to iodine vapor proved to be the best method of detecting the spots. It was the easiest to use and showed more spots than the other methods tried. Simple exposure to ultraviolet light was the second best method of spot detection. The combination of ammonia vapors and ultraviolet light did not show any improvement over the use of ultraviolet light alone. The other methods, 30% aqueous sulfuric acid, bromothymol blue spray, and p-nitroaniline spray showed no improvement over the iodine vapor method and were more difficult to use.

Four columns (Tables 7 and 8) were used in an attempt to completely resolve the compounds in the "yellow band" from the column chromatograph.

The Hi-EFF 8 BP column did not give good resolution. Even with temperature programming, it showed only five peaks. The other three columns showed no great difference in resolving power. Each of these columns showed as many as 16 peaks and the UC W-98 column showed 19 peaks.

Separation of the "yellow band" from the column chromatograph by thin-layer chromatography prior to injection into the gas chromatograph yielded improved separation. The "yellow-blue band" (high R_f) from the thin-layer plates was shown to contain two compounds by gas-liquid chromatography.

However, there were no peaks by gas-liquid chromatography which could be attributed to the "bright-blue band" (low R_f) from the thin-layer plates. It may be that the "bright-blue band" contained high-boiling compounds which did not elute under the conditions used.

The results of gas-liquid chromatography showed that at least 20 compounds were jammed into the "yellow band" from column chromatography. Nineteen of these were resolved by the UC W-98 column and there was a minimum of one in the "bright-blue band" from the thin-layer plates which did not show from the UC W-98 column making a minimum of 20 compounds. These materials must be similar in chemical nature and structure. This further increases the difficulty of separation and purification. The identification of every compound shown by gas-liquid chromatography is often impossible, mainly because of the time and difficulties involved and the minute amounts of material which can be detected.

However, the combination of thin-layer chromatography and gas-liquid chromatography produced evidence for the identity of some of the compounds. It is concluded from the thin-layer and gas-liquid chromatographic evidence that the n-hexane-soluble fraction from Douglas-fir bark contains campesterol and β-sitosterol.

A portion of the gas effluent from the gas-liquid chromatographic column was passed directly into the ion-source of the mass spectrometer. The remaining portion of the gas effluent from the column was passed through a flame ionization detector which produced an electrical signal which was recorded on an X-Y strip recorder. In this way when a peak appeared on the recorder, a portion of the compounds causing the peak was also in the mass spectrometer and a rapid scan by the mass spectrometer produced a fragmentation spectra for that compound. Thus, the gas-liquid chromatograph separated the mixture into pure compounds and the mass spectrometer yielded information as to the identity of each pure compound.

An aliquot of the trimethylsilyl ethers of the known campesterol and β-sitosterol mixture was injected into the gas chromatograph. As the peaks appeared on the recorder, a scan was made by the mass spectrometer. In this way standard mass spectra were obtained for known β-sitosterol and campesterol for reference purposes.

In the same way, the trimethylsilyl ethers of the "yellow band" were injected into the gas chromatograph. The gas-liquid chromatographic spectrum was scanned by mass spectrometry. In the mass spectrum of the trimethylsilyl ether of β-sitosterol, characteristic peaks were found at m/e 486, 396, 381, 357, 255 and 129. The base peak at m/e 129, which is a characteristic fragment for a 5-en-3-ol steroid trimethylsilyl ether was very obvious. In the fragmentation reaction the trimethylsilyl ether derivative is split into two parts, one part which is charged and the other which is neutral. The charge can reside on the m/e 129 fragment or the m/e M-129 fragment (M is the molecular ion mass). Therefore, both fragments are recorded, but usually the intensities are different.

The mass spectra were not as conclusive for campesterol as might be expected from the retention times. However, peaks at m/e 129 and 255, which are characteristic for sterols, were observed. These spectra indicated mixtures of terpenes and sterols.

The mass spectral data support the gas-liquid chromatograhic retention data as to the presence of campesterol. The ratio of campesterol to β-sitosterol is about 1 to 8 as estimated by areas under the gas-liquid chromatographic curves.

The identification of campesterol and β-sitosterol in the n-hexane-soluble fraction of Douglas-fir bark has proven very interesting because it leads to a search for other sterols and the part which they might play in bark. The physiological role of these materials is largely unknown, although Rowe (70) has suggested that β-sitosterol may have a function in cell-wall permeability.

The mass spectra also showed peaks at m/e 93 and m/e 121 which are very characteristic of terpenes and it is concluded that this particular fraction of the bark contains terpene compounds.

The acids recovered from saponified "benzene wax" could be resolved into three major families by TLC of their methyl esters. Spot 1 (R_f 0.74) migrated nearly the same distance as authentic methyl lignocerate (R_f 0.76) suggesting that it was composed of methyl esters of non-oxygenated fatty acids. Spot 2 (R_f 0.54) migrated similarly to authentic dimethyl octadecanedioate (R_f 0.57) suggesting that it was composed of dimethyl esters of dicarboxylic acids. Spot 3 (R_f 0.17) was not readily identified by standards in our possession, but further investigation suggests that it contains the methyl esters of the hydroxy acids observed by Kurth.

TLC of the neutral compounds from saponified "benzene wax" yielded two major components, spot A (R_f 0.30) and spot B (R_f 0.17). The latter spot reacted positively to steroid-detection reagents and migrated the same distance as authentic β-sitosterol. These spots could be the aliphatic alcohols and "phytosterols" observed by Kurth (72).

GLC resolved the compounds of TLC spot 1 into a number of fatty acid methyl esters (Table 9), identifications of which were based on studies with four GLC liquid phases. The ester of greatest abundance was methyl lignocerate, but substantial amounts of several other esters were present. Several of the GLC components of spot 1 remain unidentified.

The dimethyl esters identified in TLC spot 2 are listed, with their relative abundances, in Table 10. Initial identifications were based on GLC studies made with three liquid phases: SE-30, Apiezon-L, and Reoplex-400. Besides the C_{20} compound reported by Kurth (72) we found substantial amounts of other dimethyl esters.

Verification of the identities of the spot 2 dimethyl esters was obtained by tandem GLC-MS. We examined the high ends of the mass spectra of authentic dimethyl esters and of the GLC peaks in spot 2 for those m/e ion peaks characteristic of dimethyl esters of dicarboxylic acids: M, M-31, M-64, M-73, M-92, and M-105. We positively identified all of these m/e peaks for each of our authentic compounds except for C_{22}, where the peak believed to be M could not be accuratley assigned an m/e. For the C_{16} and C_{20} dimethyl esters of spot 2, we identified all the above ions; for C_{22}, all except M; for C_{24}, only M-31 and M-73. The spectra of the $C_{18:0}$ and $C_{18:1}$ dimethyl esters were of mixtures of the two, since resolution was incomplete at the mass spectrometer. However, M, M-31, M-64 and M-73 were identified for the $C_{18:0}$ dimethyl ester, and M = 340 m/c and ($M-CH_3OH$) - 308 m/e were identified, which indicates the presence of a $C_{18:1}$ dimethyl ester. The MS taken at various points on the total-ionization peak showed that the unsaturated diester emerged from the GLC first. This is consistent with the GLC studies. No obvious signs of branching were noted in any of the spectra.

Gas chromatographic analysis (SE-30) of the TLC spot thought to contain fatty alcohols showed two major components which were identified as 1-docosanol and 1-tetracosanol; smaller amounts of 1-hexadecanol, 1-octadecanol and 1-eicosanol were also present (Table 11). Earlier, Kurth (75) isolated 1-tetracosanol from the neutral fraction of saponified benzene wax.

Identification was established by co-GLC of the free alcohols of benzene wax with authentic compounds (SE-30 and Carbowax 20M) and by co-GLC (SE-30) of the alcohol acetates, methyl ethers, and TMSi ethers with the respective derivatives prepared from authentic compounds. The IR spectra (KBr) of C_{22} and C_{24} free alcohol and alcohol acetate peaks collected were identical to those of the authentic compounds. In particular, the -OH stretch bands at ca 3330 cm^{-1} present in spectra of the free alcohols were absent in spectra of the acetates; in the latter, the ester carbonyl absorption at 1745 cm^{-1} appear. Melting points of the C_{22} and C_{24} alcohols from benzene wax agreed with those of authentic compounds, and mixed m.p.'s with respective authentic compounds showed no depression.

GLC (SE-30) of TLC spot 3, thought to contain methyl esters of hydroxy acids, showed it to contain a family of six compounds for which a plot of chain length (arbitrary integer) vs. log R_t was a straight line. Five of these compounds were isolated by preparative GLC and identified as the methyl esters of ω-hydroxy acids C_{16} - C_{24} (even); not enough of the sixth compound (C_{26}?) was present to conveniently collect for positive identification. Kurth reported the presence of C_{16}, C_{20} and C_{22} hydroxy acids in benzene wax, but though he noted the unusual melting characteristics of the free hydroxy behenic acid, he did not locate the hydroxyl group (76).

Also present in the chromatogram, but unidentified, were two peaks emerging before ω-hydroxy C_{16}, and a peak (peak "2a") only partially resolved from ω-hydroxy C_{18} (Table 12). The methyl ether of peak 2a emerged after ω-hydroxy C_{18} on EGSS-X, and contained at least two major components. The GLC behavior of peak 2a, as well as the fact that it was liquid at room temperature, suggest that it may contain unsaturated C_{18} ω-hydroxy methyl esters. Kurth claims to have isolated a fraction rich in hydroxy unsaturated acids from the cork of Douglas fir bark.

Treatment of the mixed ω-hydroxy esters from benzene wax with alkali in a manner similar to saponification of the wax, followed by methylation did not produce any detectable dicarboxylic esters hence we are confident that the dicarboxylic acids identified earlier by us are not artifacts of the saponification procedure. That ω-hydroxy acid and dicarboxylic acid families of similar composition were found in benzene wax may be of biosynthetic significance.

Though not as widespread or abundant as the hydroxy acids in general, ω-hydroxy acids as constituents of plant waxes and extracts have been known for some time. The inter-esterification of the free hydroxy acids to form "estolides" occurs readily, especially in acid medium or upon heating above the melting point, and may partly explain the variable melting point observed by Kurth (76) for his hydroxy-behenic acid. Murray and Schoenfeld considered estolide formation upon acidification of a saponification mixture of ω-hydroxy acid salts enough of a problem to avoid isolating the free acids during their qualitative and quantitative analysis of the ω-hydroxy acids of carnauba wax. Estolide formation could partly explain our inability to totally account for the weight of benzene wax after isolation of neutrals, methyl esters, and "phenolic acids"; and it could explain the somewhat unreproduceable relative abundances of ω-hydroxy esters between complete isolations of the esters from the wax. The relative abundances shown in Table 12 should be considered only tentative and typical of our isolation procedure.

Identity of the ω-hydroxy acids was established by analyzing individual hydroxy methyl esters and the corresponding methoxy methyl esters collected by preparative GLC. The IR spectra (CCl_4) contained a weak, sharp band at 3620 cm^{-1}, indicating a hydroxyl group; this band was absent in the IR spectra of the corresponding ω-hydroxy methyl esters. The IR spectrum (KBr) of methyl 18-hydroxyoctadecanoate was identical to published spectrum, and the spectra of the other homologs were similar. The most conspicuous difference between homologs was the addition of one progression band in the region between 1180 and 1315 cm^{-1} for each increase of two methylene units.

The lengths of the hydrocarbon chain of the C_{16}, C_{20} and C_{22} homologs were established by reduction of the hydroxyl group and identification of the resulting fatty acids (as methyl esters) by GLC (two columns) and by GLC-MS. Hydroxyl group positions for the C_{16}, C_{20} and C_{22} homologs were established by oxidation of the hydroxyl group and identification of the resulting dicarboxylic acids (as methyl esters) by GLC (two columns) and by GLC-MS.

The mass spectra of methyl 18-methoxyoctadecanoate agreed with a published spectrum, and the spectra for the C_{16}, C_{20} and C_{22} homologs were similar. In all cases, the portions of the spectra m/e 143 and below were identical. The peak m/e 45, the strongest in the spectra, is mainly due to the fragment $CH_2OCH_3^+$ and indicates the methoxy is in the terminal (ω) position. The high m/e portions of the spectra all showed significant peaks assigned as M^+, $(M-CH_3)$, M-30, M-31, M-32, M-47, M-59, M-64 and M-92.

Finally, the melting points of the C_{16}-C_{24} ω-hydroxy esters agreed with values reported in the literature.

AMMONIATION OF BARK

I. Historical Review (80)

The use of ground bark for mulching, soil conditioning, and ornamental purposes in home gardens and elsewhere has greatly increased in recent years (1, 2, 3, 81, 82). A major consideration in these applications is to ensure that the addition of bark does not lower the nitrogen content of the soil. Bark contains very little nitrogen and the microorganisms which bring about its decomposition tend to compete with plant roots for the available nigrogen (83, 84). According to Bollen (85) the fortification of bark with nitrogen is vital to the successful use of bark on or in the soil. Treatments include chemical processing, mechanical mixing with nitrogen fertilizers, aging, composting and combinations of treatments. The work herein reported is concerned with fortification by chemical processing and with gaseous ammonia in particular.

There have been several reports on the subject (3, 83, 84, 85, 86, 87). Aspitarte (86) and Bollen and Glennie (83) exposed Douglas-fir bark to anydrous ammonia in a closed screw conveyor system. Bollen and Glennie (84) also sprayed ground bark with aqueous ammonia, nitric acid, an aqueous solution of urea, and an aqueous solution of urea and phosphoric acid. Simpler procedures have involved covering a pile of bark with a polyethylene sheet and passing gaseous ammonia through it from a perforated pipe or hose (85, 88).

Our objectives in the present study included a determination of the amount of nitrogen retained by Douglas-fir bark after treatment with gaseous ammonia under controlled conditions. We also wished to ascertain how readily this nitrogen was extracted with selected solvents and if any nitrogen remained after exhaustive extraction. We investigated the extracts to determine if the ammoniation altered the chemical compounds which are extracted by organic solvents and water. Since these compounds are most likely to be leached into the soil, we particularly wanted to determine if any known harmful products resulted.

II. Experimental (80)

A. Sample Preparation

Samples of bark from Douglas-fir [*Pseudotsuga* *menziesii* (Mirb.) Franco] were collected near Corvallis, Oregon. They were thoroughly mixed, ground in a ball mill to a size suitable for research investigations and air dried; moisture content, 15.8% (oven at 110°C overnight); nitrogen content, 0.46% (Kealdahl).

B. Bark Extraction

An aliquot (21.04 g) of the bark was successively extracted in a Soxhlet apparatus (50 exchanges of solvent) with *n*-hexane, benzene, diethyl ether, 95% ethanol and hot water. An ultraviolet spectrum of each extract was obtained over a range of 230 nm to 350 nm. Each extract was concentrated under vacuum on a rotary evaporator, dried in an oven at 110°C overnight, and the solids weighed.

C. Treatment with Gaseous Ammonia

An aliquot (233.26 g) of the air-dried bark was loosely packed into a glass tube to provide a sample 4.6 cm in diameter and 100.0 cm in length. Typical conditions for ammonia treatment were: tank gauge pressure, 10 psi, time of gaseous ammonia flow, 2 hr; total time in ammonia atmosphere, 17 hr; maximum temperature in sample, 68°C; nitrogen content of air-dried bark after treatment, 4.08%.

An aliquot (25.00 g) of the treated bark after air drying was successively extracted with *n*-haxane, benzene, diethyl ether, 95% ethanol and hot water and the extracts investigated as described above for those from the original bark. The nitrogen content of the bark residue after extraction was 2.98%.

III. Results and Discussion (80)

The sample of Douglas-fir bark used in the present treatment contained 0.14% nitrogen. This is similar to the 0.11% and 0.16% reported by Bollen and Glennie (86). After treatment with gaseous ammonia the nitrogen content increased to 4.08% which is in the range of 3.71% and 4.27% obtained by Bollen and Glennie (83) for bark ammoniated under selected conditions. This amount of nitrogen has been shown (83, 84) to be more than adequate for the prevention of nitrogen starvation in soil.

Ammoniation of finely ground bark (passed a screen of 32 meshes to the inch) resulted in a nitrogen content to 2.34%. The lower nitrogen retention was attributed to a more solid packing of the fine particles in the treating tube so that the ammonia gas may not have diffused freely through the sample. A loose packing of the bark or an increased treating time would be expected to increase the nitrogen content. This illustrates the flexibility of ammoniation with gaseous nitrogen. By careful control of treating conditions, it is possible to tailor the nitrogen content of bark to the desired content. Bollen and Glennie (83) also demonstrated this control. They obtained nitrogen contents ranging from 1.68% to 4.27% by changing the treating conditions.

The amount of solids extracted in each of the organic solvents was not significantly altered by treatment with gaseous ammonia (Table 13). However, the hot water extraction of ammoniated bark resulted in an increased solid dissolution of 3.5 times for coarse bark and 3.2 times for finely ground bark when compared to untreated bark. Ammoniation is known to increase the pH of bark (83, 84) which could bring about hydrolysis of some of the polyphenolic compounds so that they would become water soluble. The data (Table 13) indicate that there would be a more immediate release of leachable solids into the soil from ammoniated bark than from untreated bark. However, there appears to be no evidence of toxic materials from ammoniated bark. Bollen and Glennie (83, 84) and Bollen and Lu (87) report that tannins, resins, and other readily extracted compounds from treated and untreated bark have no toxic effects on plants when added to the soil. The conditions of extraction used in the present study are considered to be exhaustive and it appears that the early rate of leaching would not be continuous but would level off to a slow dissolution of the bark due to microbial action.

Table 13 also shows that grinding the bark to a small screen size has little or no effect upon the amounts of solids extracted by each solvent. Therefore, whether the bark is used as chunk-sized decorative bark, or ground and used as a mulch has little effect upon the immediate dissolution of solids. However, the immediate leaching of solids should not be confused with microbial decomposition for in the latter case bark size has a considerable effect. According to Bollen (85) the finer the bark particles the more rapidly it is decomposed.

The bark residue from the exhaustive extraction with organic solvents and hot water analyzed for 2.98% nitrogen. These results support the findings of Bollen and Glennie (83, 84) that a part of the nitrogen in ammoniated bark is immediately available but that a substantial part is slowly released only through normal decomposition. In many soil uses this dual effect is very desirable and beneficial.

The ultraviolet spectra of the extracts showed that little if any chemical changes occurred in the extractable materials because of ammoniation (Table 14). Only the major peaks and shoulders are reported, but in general there were little differences in the spectra. Although the wavelength range included only the ultraviolet region, it is expected that major changes in the leachable compounds would have been detected. Indications are that chemical changes which might have occurred took place in those compounds which were not soluble.

BIBLIOGRAPHY

1. Harkin, J. M. and J. W. Rowe. Bark and its possible uses. USDA Forest Service, Research Note FP:-0910, October 1969.

2. Bark Committee of the Forest Products Research Society. Marketing and selling bark products. Ed. by Jean Mater, R. Martin, D. Williams, R. Sarles, F. Lamb, and R. Allison, Forest Products Research Society, Madison, WI. 1969.

3. Hall, J. Alfred. Utilization of Douglas-fir bark. Pacific Northwest Forest and Range Experiment Station, Forest Service, U. S. Department of Agriculture, Portland, OR. 1971.

4. Grillos, S. J. Structure and development of the bark of Douglas-fir Pseudotsuga menziesii (Mirb.) Franco. Doctoral thesis. Corvallis, Oregon State University. 1956. 67 numb. leaves.

5. Grillos, S. J. and F. H. Smith. The secondary phloem of Douglas-fir. Forest Science 5:377-388. 1959.

6. Chang, Y. Bark structure of North American conifers. (U. S. Dept. of Agriculture, Technology Bulletin 1095). 1954. 86 p.

7. Ross, W. D. and R. L. Krahmer. Some sources of variation in structural characteristics of Douglas-fir bark. Wood and Fiber 3:35-46. 1971.

8. Kiefer, H. J. and E. F. Kurth. The chemical composition of the bast fibers of Douglas-fir bark. Tappi 36:14-19. 1953.

9. Segall, G. H. and C. B. Purves. Chemical composition of wood barks. Pulp and Paper Magazine of Canada 47:149-161. 1946.

10. Kurth, E. F. The chemical composition of bark. Chemical Reviews 40:33-49. 1947.

11. Aspinall, G. O. Structural chemistry of the hemicelluloses. In: Advances in carbohydrate chemistry. Vol. 14. Ed. by M. L. Wolfrom. New York, Academic Press, Inc. 1959. p. 429-433.

12. Timell, T. E., C. P. Glaudemans and J. K. Gillham. Recent studies on the polysaccharices of white birch and other hardwoods. Tappi 42:623-634. 1959.

13. Hamilton, J. K. and N. S. Thompson. A comparison of the carbohydrates of hardwoods and softwoods. Tappi 42:752-760. 1959.

14. Timell, T. E. Wood hemicelluloses. In: Advances in carbohydrate chemistry. Vol. 19. Ed. by M. L. Wolfrom. New York, Academic Press, Inc. 1964. p. 247-302.

15. Whistler, R. L. and E. L. Richards. Hemicelluloses. In: The Carbohydrates Chemistry and Biochemistry, Vol. IIA, 2nd edition. Ed. by W. Pigman and D. Horton. New York, Academic Press, Inc. 1970. 469 p.

16. Graham, H. M. and E. F. Kurth. Constituents of extractives from Douglas-fir. Industrial Engineering Chemistry 41:409-414. 1949.

17. Holmes, G. W. and E. F. Kurth. The chemical composition of the newly formed inner bark of Douglas-fir. Tappi 44:893-898. 1961.

18. Cleland, J. E. and W. R. Fetzer. Determination of total solids in glucose syrup. Industrial and Engineering Chemistry. Analytical Edition 13:858-860. 1941.

19. Timell, T. E., C. P. Glaudemans and A. C. Currie. Spectrophotometric method for determination of sugars. Analytical Chemistry 28: 1916-1920. 1956.

20. Timell, T. E. Isolation of polysaccharides from the bark of gymnosperms. Svensk papperstidning 64:651-661. 1961.

21. Browning, B. L. Methods of wood chemistry. Vol. I. New York, Interscience Publishers. 1967. 384 p.

22. Laver, M. L., D. F. Root, F. Shafizadeh and J. C. Lowe. An improved method for the analysis of the carbohydrates of wood pulps through refined conditions of hydrolysis, neutralization, and monosaccharide separation. Tappi 50:618-622. 1967.

23. Black, R. J., R. LeShange and G. Zweig. Paper chromatography, a laboratory manual. New York, Academic Press, Inc. 1952. 195 p.

24. Clark, J. M., Jr. Experimental biochemistry. San Francisco, CA, W. H. Freeman and Co. 1964. 228 p.

25. Hough, L. and J. K. N. Jones. Chromatography on paper. In: Methods in carbohydrate chemistry. Vol. I. Ed. by R. L. Whistler, M. L. Wolfrom. New York, Academic Press. 1962. 589 p.

26. Piper, C. V. and L. J. Bernardin. A spectrophotometric method for the chromatographic analysis of sugars. Tappi 41:16-18. 1958.

27. Miles Laboratories, Inc. HT-1000. Product Information. Elkhart, Indiana. No date.

28. Miles Laboratories, Inc. Diazyme, an amyloglucosidase enzyme for conversion of starch and/or dextrins to glucose. Technical Bulletin No. 9-245. Elkhart, Indiana. 1963.

29. Wolfrom, M. L. and D. L. Patin. Carbohydrates of the coffee bean. IV. An arabinogalactan. Journal of Organic Chemistry 30:4060-4063. 1965.

30. Abdel-Ahker, M., J. K. Hamilton and F. Smith. The reduction of sugars with sodium borohydride. Journal of the American Chemical Society 73:46-91-4692. 1961.

31. Borchardt, L. G. and C. V. Piper. A gas chromatographic method for carbohydrates as alditol-acetates. Tappi 53:257-260. 1970.

32. Albersheim, P., D. J. Nevins, P. D. English, and A. Karr. A method for the analysis of sugars in plant cell-wall polysaccharides by gas-liquid chromatography. Carbohydrate Research 5:300-345. 1967.

33. Sloneker, J. H. Gas-liquid chromatography of alditol acetates. In: Methods in Carbohydrate Chemistry. VI. General Carbohydrate Methods. Ed. by R. L. Whistler and J. N. BeMiller. New York, Academic Press. 1972. 603 p.

34. Paech, K. and M. V. Tracey. Moderne Methoden Per Pflanzen-analyse (modern methods of plant analysis). 1:468-473. 1956.

35. Wolfrom, M. L. and D. L. Patin. Carbohydrates of the coffee bean. IV. An arabinogalactan. Journal of Organic Chemistry 30:4060-4063. 1965.

36. Vogel, I. A. Textbook of practical organic chemistry including qualitative organic analysis. Third ed., New York, Longmans, Green and Co. 1956. 1188 p.

37. Clark, John N. Experimental biochemistry. San Francisco, CA, W. H. Freeman and Co. 1964. 228 p.

38. Pische, S. Color reactions of hexuronic acids. In: Methods of Carbohydrate Chemistry, Vol 1. Ed. by R. L. Whistler and M. L. Wolfrom, New York, Academic Press. 1962. 589 p.

39. Jayme, G. and H. Knolle. Paper chromatography of sugar mixtures on glass-fiber papers. Angewande Chemie 68:243-246. 1956.

40. Partridge, S. M. Aniline hydrogen phthalate as a spraying reagent for chromatography of sugars. Nature 164:443. 1949.

41. Wolfrom, M. L., M. L. Laver and D. L. Patin. Carbohydrates of the coffee bean. II. Isolation and characterization of a mannan. Journal of Organic Chemistry 26:4533-4535. 1961.

42. Falconer, E. L. and G. A. Adams. The aldobiouronic acids of hemicellulose B of oat hulls. Canadian Journal of Chemistry 34: 338-344. 1956.

43. Acid Insoluble Lignin in Wood Pulp. Tappi Standard T222 M-24.

44. Browning, B. L. and L. O. Bublitz. The ioslation of holocellulose from wood. Tappi 36:452-458. 1953.

45. Goldschmid, Otto. Ultraviolet spectra. In: Lignins, Occurrence, Formation, Structure and Reactions. Ed. by K. V. Sarkanen and C. H. Ludwig. New York, Wiley-Interscience. 1971. 916 p.

46. Wolfrom, M. L. and J. V. Karabinos. The identification of aldose sugars by their mercaptal acetates. Journal of the American Chemical Society 67:500-501. 1945.

47. Bates, F. J. and Associates. Polarimetry, saccharimetry and the sugars. National Bureau of Standards Circular C440. 1942. p. 488.

48. Whistler, R. L. and J. N. BeMiller. α-D-xylose. I. Isolation from corn cobs or xylan. In: Methods in Carbohydrate Cehmistry, Vol. 1. Ed. by R. L. Whistler and M. L. Wolfrom. New York, Academic Press. 1962. 589 p.

49. Isbell, H. S. and H. L. Frush. α-D-Mannose from ivory-nut meal. In: Methods in Carbohydrate Chemistry, Vol. 1. Ed. by R. L. Whistler and M. L. Wolfrom. New York, Academic Press. 1962. 589 p.

50. Fales, H. A. and F. Kenny. Inorganic quantitative analysis. New York, Appleton-Century-Crofts, Inc. 1939. 717 p.

51. Fisher, R. B. Quantitative chemical analysis. Second ed. Philadelphia and London, W.B., Saunders Co. 1964. 501 p.

52. Hodge, J. E. and B. T. Hofreiter. Determination of reducing sugars and carbohydrates. In: Methods in Carbohydrate Chemistry, Vol. I. Ed. by R. L. Whistler and M. L. Wolfrom. New York, Academic Press. 1962. 589 p.

53. Sawardeker, J. S., J. H. Soneker and A. Jeanes. Quantitative determination of monosaccharides as their alditol acetates by gas liquid chromotography. Analytical Chemistry 12:1602-1603. 1965.

54. Langlois, David L. myo-Inositol from corn steep water or calcium phytaten. In: Methods in Carbohydrate Chemistry, Vol. II. Ed. by R. L. Whistler and M. L. Wolfrom. New York, Academic Press. 1963. 572 p.

55. Beélik, A., R. J. Conca, J. K. Hamilton and E. V. Partlow. Selective extraction of hemicelluloses from softwoods. Tappi 50:78-81. 1967.

56. Technical Association of the Pulp and Paper Industry. Ash in pulp. Tappi Standards and Suggested Methods. T211 M-58. New York. 1958.

57. Willard, H. H., L. L. Merritt, Jr. and J. A. Dean. Instrumental methods of analysis. Fourth edition. Princeton, N.J., Van Nostrand Co., Inc. 1965. 784 p.

58. Browning. B. L. Methods of wood chemistry, Vol. II. New York, Interscience Publishers. 1967. p. 384-882.

59. Song, M. J. The structure of a xylan from Tilia americana L. Master's thesis. Syracuse, N.Y., State University College of Forestry at Syracuse University. 1970. 97 numb. leaves.

60. Technical Association of the Pulp and Paper Industry. Cupriethylenediamine disperse viscosity of pulp. Tappi Standards and Suggested Methods. T230 SU-66. New York. 1966.

61. Sears, K. D., A. Beélik, R. L. Casebier, R. J. Engen, J. K. Hamilton and H. L. Hergert. Southern pine prehydrolyzates: characterization of polysaccharides and lignin fragments. Journal of Polymer Science Part C:425-443. 1971.

62. Lai, Y. Z. Kinetics of degradation of carbohydrates. Doctoral thesis. Seattle, University of Washington. 1968. 97 numb. leaves.

63. Lai, Y. Z. and K. V. Sarkanen. Kinetic study on the alkaline degradation of amylose. Journal of Polymer Science, Part C, 28:15-26. 1969.

64. Barnett, A. J. G. and G. A. Tawab. A rapid method for the determination of lactose in milk and cheese. Journal of Scientific Food Agriculture 8:437-441. 1957.

65. Dubois, M., K. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith. Colorimetric method for determination of sugars and related substances. Analytical Chemistry 28:350-356. 1956.

66. Samuelson, O. and A. Wennerblom. Degradation of cellulose by alkali cooking. I. Formation of carboxyl groups. Svensk Papperstidning 57:827-830.

67. Boggs, L., L. S. Cuendet, I. Ehrenthal, R. Koch and F. Smith. Separation and identification of sugars using paper chromatography. Nature 166:520-521. 1950.

68. Hough, L., J. K. N. Jones and W. H. Wadman. Quantitative analysis of mixtures of sugars by the method of partition chromatography. Part V. Improved methods for the separation and dectection of the sugars and their methylated derivatives on the paper chromatogram. Journal of the Chemical Society 1702-1706. 1950.

69. Partridge, S. M. Aniline hydrogen phthalate as a spraying reagent for chromatography of sugars. Nature 164:443. 1949.

70. Wolfrom, M. L. and D. L. Patin. Isolation and characterization of cellulose in the coffee bean. Agriculture and Food Chemistry 12:376-377. 1964.

71. Kurth, E. F. and H. J. Kiefer. Wax from Douglas-fir bark. Tappi 33:183-186. 1950.

72. Kurth, E. F. The composition of the wax in Douglas-fir bark. Journal of the American Chemical Society 72:1685-1686. 1950.

73. Fieser, L. F. Experiments in organic chemistry. Third ed. Boston, D. C. Heath and Co. 1957. 353 p.

74. Fieser, L. F. and Mary Fieser. Natural products related to phenantherene. Third ed. New York, Reinhold Publ. Co. 1949. 704 p.

75. Kurth, E. F. The chemical composition of conifer bark waxes and corks. Tappi 50:253-257. 1967.

76. Hergert, H. L. and E. F. Kurth. The chemical nature of the cork from Douglas-fir bark. Tappi 35:59-66. 1952.

77. Kiefer, H. J. and E. F. Kurth. The chemical composition of the bast fibers of Douglas-fir bark. Tappi 36:14-19. 1953.

78. Loveland, P. M. and M. L. Laver. Monocarboxylic and Dicarboxylic acids in the benzene extract of Pseudotsuga menziesii bark. Phytochemistry 11:430-432. 1972.

79. Loveland, P. M. and M. L. Laver. ω-Hydroxy fatty acids and fatty alcohols from Pseudotsuga menziesii bark. Phytochemistry 11: 3080-3081. 1972.

80. Laver, M. L., J. V. Zerrudo and Harvey Aft. Treatment of Douglas-fir bark with gaseous ammonia. Forest Products Journal 22:82-83. 1972.

81. Mater, Jean, ed. Marketing bark agricultural and horticultural products. Forest Products Research Society, Madison, WI. 1970.

82. Mater, Jean. Utilization of bark in highway landscaping. Forest Products Journal 21:17-20. 1971.

83. Bollen, W. B. and D. W. Glennie. Sawdust, bark and other wood wastes for soil conditioning and mulching. Forest Products Journal 11:38-46. 1961.

84. Bollen, W. B. and D. W. Glennie. Fortified bark for mulching and soil conditioning. Forest Products Journal 13:209-215. 1963.

85. Bollen, W. B. Properties of tree barks in relation to their agricultural utilization. Research Paper PNW-77, U. S. Dept. of Agric., For. Serv., Pacific NW For. and Range Exp. Sta., Portland, Oregon. 1969.

86. Aspitarte, T. R. Availability of nitrogen in ammoniated bark used as a soil amendment. Doctoral thesis, Oregon State University, Corvallis, Oregon. 1959.

87. Bollen, W. B. and K. C. Lu. Douglas-fir bark tannin decomposition in two forest soils. Research Paper PNW-85, U. S. Dept. of Agric., For. Serv., Pacific NW For. and Range Exp. Sta., Portland, Oregon. 1969.

88. Ticknor, R. L. and J. L. Ricard. Oregon State University, North Willamette Exp. Sta., Aurora, Oregon. Personal communication. 1971.

Table 1. Raw material for pelleting trials

Pure Barks	Mixtures
1. Douglas fir	1 + 2
2. western hemlock	2 + 8
3. western larch	1 + 2 + 3 + 4 + 7
4. ponderosa pine	Douglas fir bark + shavings
5. lodgepole pine	Douglas fir bark + hog fuel
6. sugar pine	Douglas fir bark + hog fuel + shavings
7. western white pine	Douglas fir bark + sawdust
8. white fir	Douglas fir bark + sanderdust
9. noble fir	Douglas fir bark + fertilizer
10. silver fir	western hemlock bark + fertilizer
11. grand fir	chemically extracted Douglas fir bark
12. western redcedar	western redcedar bark plus woody material
13. Sitka spruce	mixed northern hardwood bark from Maine
14. Engelmann spruce	mixed eastern softwood bark from Maine
15. red alder	mixed eastern softwood bark + sawdust
	southern oak bark + woody material from Tennessee

Table 2. Bulk densities and compression factors for some pelleted barks.

Bark species	Pellet diameter (inches)	Bulk density (lb/cu ft) Before pelleting	Bulk density (lb/cu ft) After pelleting	Compression factor
Douglas fir	1/2	14.2	39.3	2.8
Douglas fir bast fibers	1/2	13.0	49.4	3.8
Douglas fir fines	3/16	13.4	45.7	3.4
Western hemlock	1/2	14.9	45.2	3.0
Western hemlock	1/4	14.9	43.0	2.9
Western hemlock	3/16	14.9	49.6	3.3
Ponderosa pine	1/2	12.6	42.3	3.4
Ponderosa pine	1/4	12.6	38.4	3.0
Lodgepole pine	1/4	11.1	37.6	3.4
Lodgepole pine	3/16	11.1	42.0	3.8
Silver fir	1/2	14.0	45.8	3.8
Silver fir	1/4	14.0	44.9	3.2
Silver fir	3/16	14.0	46.0	3.3
White fir	1/2	14.7	38.9	2.6
White fir	1/4	14.7	40.3	2.7
White fir	3/16	14.7	35.6	2.4
Grand fir	1/2	14.9	37.4	2.5
Grand fir	1/4	14.9	30.4	2.0
Red alder	1/2	22.3	46.7	2.1
Red alder	3/16	22.3	46.2	2.1
Mixed eastern softwoods	1/2	15.9	47.5	3.0

Table 3. Partial list, recipients of bark pellet samples

	Company	Location
1.	Dant and Russell, Inc.	Portland, Oregon
2.	Sigma Plastics	Portland, Oregon
3.	Grant & Roth Plastics	Hillsboro, Oregon
4.	Colorado State University	Ft. Collins, Colorado
5.	Small Business Administration	Portland, Oregon
6.	California Pellet Mill Company	Portland, Oregon
7.	Bohemia, Inc.	Eugene, Oregon
8.	Fruit Growers' Supply Company	Los Angeles, California
9.	Sequoia Forest Products	Dinuba, California
10.	DiGiorgio Corporation	San Francisco, California
11.	MacMillan Bloedel	Vancouver, B. C., Canada
12.	Architectural Short Course Display	Portland, Oregon
13.	MEC Company	Neodesha, Kansas
14.	Publishers Paper Company	Portland, Oregon
15.	Western Wood Fibre	Eugene, Oregon
16.	U. S. Plywood-Champion Papers	Eugene, Oregon
17.	U. S. Forest Service	Washington, D. C.
18.	Oregon Timber Products Company	Albany, Oregon
19.	Reydco Trading Company	Eugene, Oregon
20.	University of Maine	Farmington, Maine
21.	Borden Company	Springfield, Oregon
22.	Ellingson Timber Company	Baker, Oregon
23.	U. S. Forest Products Laboratory	Madison, Wisconsin
24.	Oregon Lumber Export Company	Portland, Oregon

25.	Pacific Keynon Corporation	Los Angeles, California
26.	Oxford Paper Company	Rumford, Maine
27.	National Distillers Products Company	Memphis, Tennessee
28.	Canadian Car (Pacific)	Vancouver, B. C., Canada
29.	H. J. Stoll & Sons	Portland, Oregon
30.	W. R. Grace & Company	Halsey, Oregon
31.	Princeton Turf Farms	Cranbury, New Jersey
32.	Potlatch Forests, Inc.	Lewiston, Idaho
33.	University of Washington	Seattle, Washington
34.	FAO	Rome, Italy
35.	Agricultural Engineering Dept., OSU	Corvallis, Oregon
36.	Bear River Lumber Company	Logan, Utah
37.	Hercules, Inc.	San Francisco, California
38.	Agricultural Chemistry Dept., OSU	Corvallis, Oregon
39.	B. P. John Furniture Company	Portland, Oregon
40.	Crown-Zellerbach Corporation	Portland, Oregon
41.	Koppers Company	Los Angeles, California
42.	American Forest Products Company	Martell, California
43.	Peco, Inc.	Portland, Oregon
44.	Head Groundskeeper, OSU	Corvallis, Oregon
45.	NASA	Moffett Field, California
46.	Chemistry Dept. Brigham Young Univ.	Provo, Utah
47.	George D. Ward & Associates	Portland, Oregon
48.	Weyerhaeuser Company	Longview, Washington
49.	Institute for Wood Biology & Wood Preservation	Hamburg, West Germany
50.	Collier Carbon & Chemical Company	Salem, Oregon

51.	Unalit S. A.	St. Usage, France
52.	Wood Products Service Company	Memphis, Tennessee
53.	Department of Forestry	Salem, Oregon
54.	Ekono	Helsinki, Finland
55.	Monsanto Company	Eugene, Oregon
56.	University of Denver	Denver, Colorado
57.	Incentive, AB	Stockholm, Sweden
58.	Agricultural Service Corporation	Salem, Oregon
59.	Canadian Forestry Service	Victoria, B. C., Canada

Table 4. Composition of bast fibers and Douglas-fir wood.

	Fiber[b]	Wood[a]
Ether soluble	2.92	1.32
Alcohol soluble	8.65	5.46
Hot water soluble	2.58	2.82
Sum of three extractives	14.15	9.60
Values based on oven-dry extractive-free material		
Ash	0.60	0.17
Lignin	44.80	30.15
Holocellulose	54.58[b]	71.40
Pentosans	8.62	10.11
Methoxyl group	3.89	4.75
Acetyl group	2.39	0.59
Uronic acid anhydride	4.62	2.80
Methoxyl on lignin	7.16	15.20

[a] Values in percent of oven-dry bark.

[b] Corrected for lignin content.

Table 5. Mixtures of alditol acetates for determination of an "Instrument K Factor"

Sugar	Weight in mg				
Myo-inositol hexaacetate	10.0	10.0	10.0	10.0	10.0
Glucitol hexaacetate	8.0	12.0	16.0	20.0	24.0
Galactitol hexaacetate	0.3	6.5	0.6	7.5	0.9
Mannitol hexaacetate	1.4	2.1	2.8	3.5	6.2
Arabinitol pentaacetate	0.4	0.6	0.8	1.0	1.2
Xylitol pentaacetate	1.1	1.65	2.2	2.75	3.3

Table 6. Extractives content of the bast fibers of Douglas-fir bark.

Solvent	Yield %[a]	Nature
n-Hexane	1.59	Light yellow "wax"
Benzene	0.95	Brown "wax"
Diethyl ether	0.38	Dihydroquercetin
Hot water	9.61	Tannin, carbohydrate, etc.
Ethanol	0.96	Phlobaphine

[a] Percentages based on oven-dry weight of bast fibers.

Table 7. Columns used in gas-liquid chromatography.

Col. No.	Length ft.	O.D. in.	Liquid coating	Concentration	Solid support	Mesh	Instrument
1	12	1/8	SE-52	10 wt. %	Gas Chrom. W.	60/80	Aerograph 200
2	6	1/8	OV-17	3 wt. %	Gas Chrom. Q.	100/120	Hewlett-Packard
3	6	1/8	US W-98	10 wt. %	Silicone Gum	80/100	Hewlett-Packard
4	6	1/8	Hi-EFF 8 BP	3 wt. %	Gas Chrom. Q.	100/120	Hewlett-Packard

[a] Outside diameter

Table 8. Conditions for separation of the "Yellow Band" by gas-liquid chromatography.

Sample	Detector temp. °C	Injection port temp. °C	Column	Column temp. °C	Flow rate ml/min	Range setting	Attenuation setting	Instrument
Silylated Mixture	265	277	SE-52	Isothermal 255	60	1	4	Aerograph
Mixture	265	277	SE-52	Isothermal 255	60	1	4	Aerograph
Mixture	270	275	UC W-98	Isothermal 275	28	10^2	16	Hewlett-Packard
Mixture	255	235	UC W-98	Program 148~300 2°/min	28	10^2	16	Hewlett-Packard
Mixture	278	272	OV-17	Isothermal 255	30	10^2	16	Hewlett-Packard
Mixture	260	245	OV-17	Program 170~300 2°/min	30	10^2	16	Hewlett-Packard
Mixture	275	270	Hi-Eff 8 BP	Program 140~300 30°/min	30	10^2	16	Hewlett Packard

Table 9. Monocarboxylic acids[1] in Douglas-fir bark "benzene wax".

Acid[2]	Retention time SE-30, 215°	Percent relative abundance
Palmitic	1.3	0.9
(Unident. sat'd.)	1.6	0.3
(C_{18} unsat'd.)	2.2	3.1
Stearic	2.4	0.4
(Unident. unsat'd.)	3.1	0.8
"	3.8	trace
"	4.5[3]	0.4
"	4.5[3]	3.3
Arachidic	4.5	2.5
n-Heneicosanoic	6.4	trace
Behenic	8.9	22.3
n-Tricosanoic	12.1	0.7
Lignoceric	17.0	51.7
n-Pentacosanoic	23.0	0.5
Cerotic	31.7	12.7
(N-Heptacosanoic)	----[4]	trace
(Montanic)	59.3	0.3
		99.9

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[1] Identified by GLC of methyl esters in TLC spot 1.

[2] Named compounds in parentheses, "(---)", denote tentative identifications.

[3] These peaks were resolved from methyl arachidate at 195°.

[4] Observed in a temperature-programmed run on SE-30, and isothermally on Apiezon-L.

Table 10. Dicarboxylic acids[1] in Douglas-fir bark "benzene wax".

Acid	Percent relative abundance
Hexadecanedioic	36.3
Octadec-?-enedioic[3]	24.8[4]
Octadecanedioic	14.6
Eicosanedioic	14.3
Docosanedioic	8.7
Tetracosanedioic	1.3
	100.0

---

[1] Identified by GLC of dimethyl esters in TLC spot 2 and verified by tandem GLC-MS.

[2] Estimated from SE-30 data.

[3] Location of double bond not established.

[4] includes small, unresolved shoulder.

Table 11. Aliphatic alcohols from benzene wax.

Acid	Percent relative abundance[1]
1-hexadecanol	trace
1-octadecanol	4.0
1-eicosanol	3.6
1-docosanol	45.0
1-tetracosanol	47.5
	100.1

---

[1] Based on peak areas from GLC of acetate derivatives.

Table 12. GLC of TLC spot 3 containing methyl esters of ω-hydroxy acids.

GLC peak (SE-30)	Acid	R_t[1] (min)	Percent relative abundance[2]
00	unident.	0.8	1.7
0	unident.	2.7	7.8
1	16-hydroxyhexadecanoic	9.5	25.6
2a	unident.	14.1	7.1
2	18-hydroxyoctadecanoic	15.1	7.1
3	20-hydroxyeicosanoic	21.4	18.0
-	unident.	22.9	2.0
4	22-hydroxydocosanoic	27.8	23.6
5	24-hydroxytetracosanoic	33.9	5.9
6	(26-hydroxyhexaconsanoic)[3]	41.1	1.3

---

[1] For temp. programed run 180-248°.

[2] Calc. from peak areas, uncorrected for detector response.

[3] Tentative identification.

Table 13. Solvent extraction yields from bark

Solvent	Percent Yield[1]		
	Untreated bark	NH_3-treated bark[2]	NH_3-treated bark (32 mesh)[3]
n-Hexane	4.67	4.63	4.16
Benzene	2.70	2.95	3.92
Diethyl ether	1.81	2.21	1.19
Ethanol	6.38	7.13	6.06
Hot water	2.20	7.60	7.00

[1] Percentages based on dry weight.

[2] NH_3 represents gaseous ammonia.

[3] Passed a screen of 32 meshes to the inch.

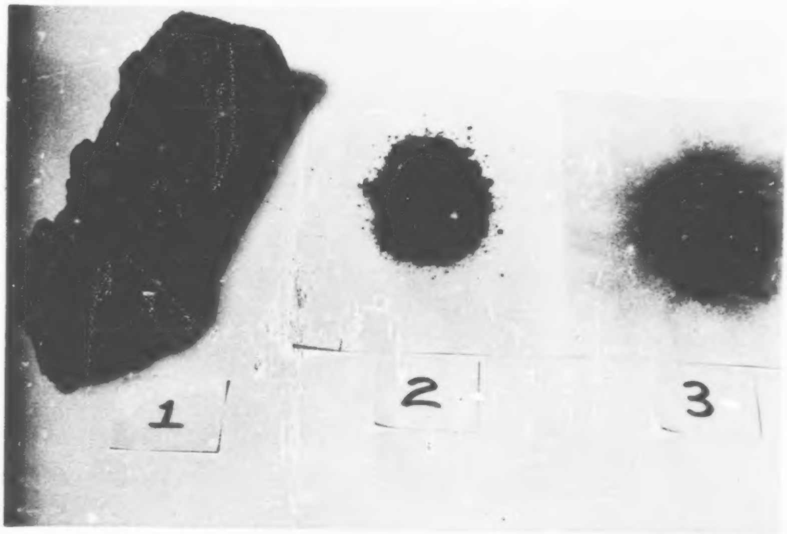
Table 14. Ultraviolet absorption of bark extracts.

Extraction solvent and bark sample	Major peak nm	Shoulder nm
n-Hexane		
Untreated bark	264	228
NH_3-treated bark[1]	264	330
NH_3-treated bark (32 mesh)[2]	264	229
Benzene		
Untreated bark	262	---
NH_3-treated bark	263	---
NH_3-treated bark (32 mesh)	265	---
Diethyl ether		
Untreated bark	254	---
NH_3-treated bark	257	---
NH_3-treated bark (32 mesh)	258	---
Ethanol		
Untreated bark	261	---
NH_3-treated bark	265	---
NH_3-treated bark (32 mesh)	264	---
Hot water		
Untreated bark		
NH_3-treated bark	249	---

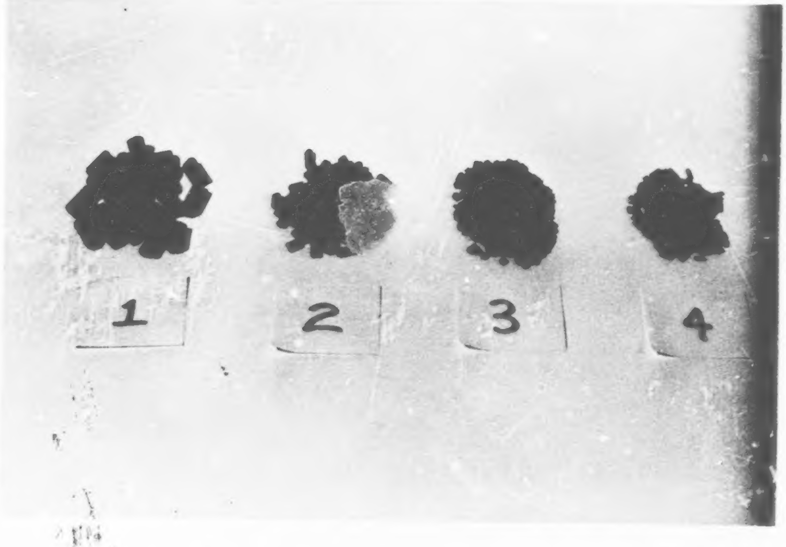
[1] NH_3 represents gaseous ammonia.

[2] Passed a screen of 32 meshes to the inch.

Figure 1

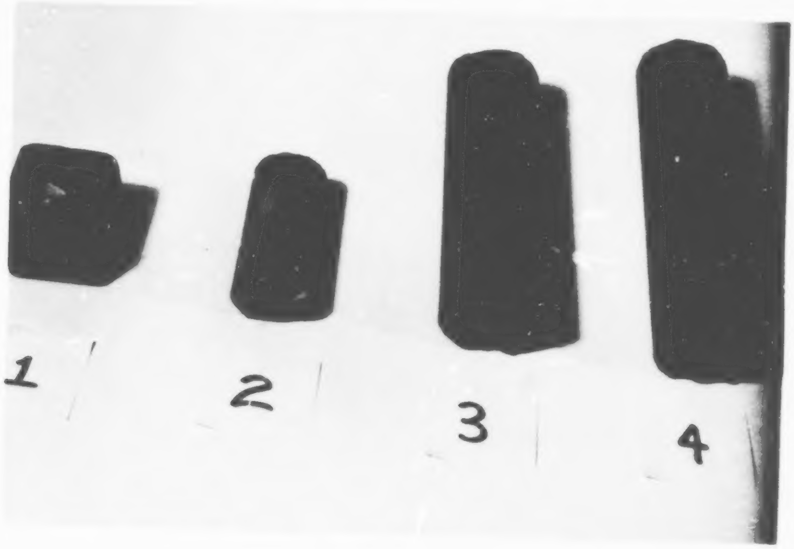


1. Douglas fir bark from veneer logs.
2. Through hammermill containing 1/4-inch screen.
3. Reground to pass 20 mesh screen.

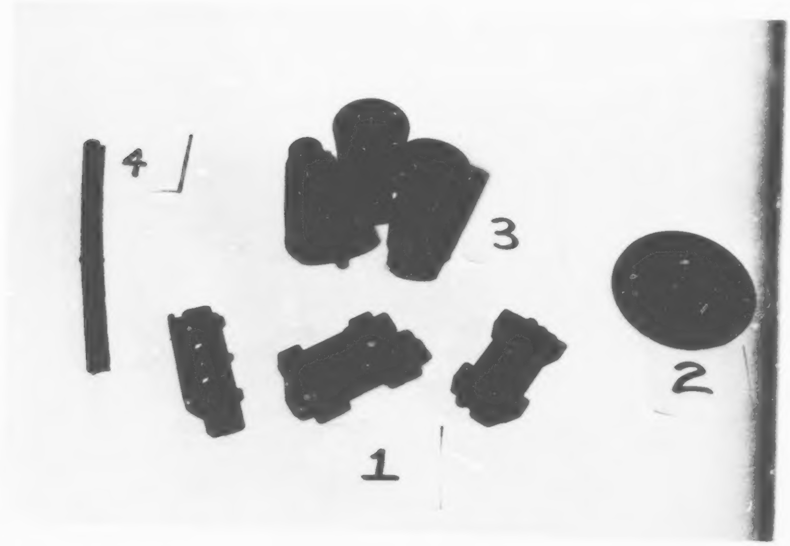


1. Pelleted Sitka spruce bark, 1/2-inch die.
2. Pelleted red alder bark, 1/4-inch die.
3. Pelleted Douglas fir bark with fertilizer added, 3/16-inch die.
4. Pelleted Douglas fir bark. 1/10-inch die.

Figure 2

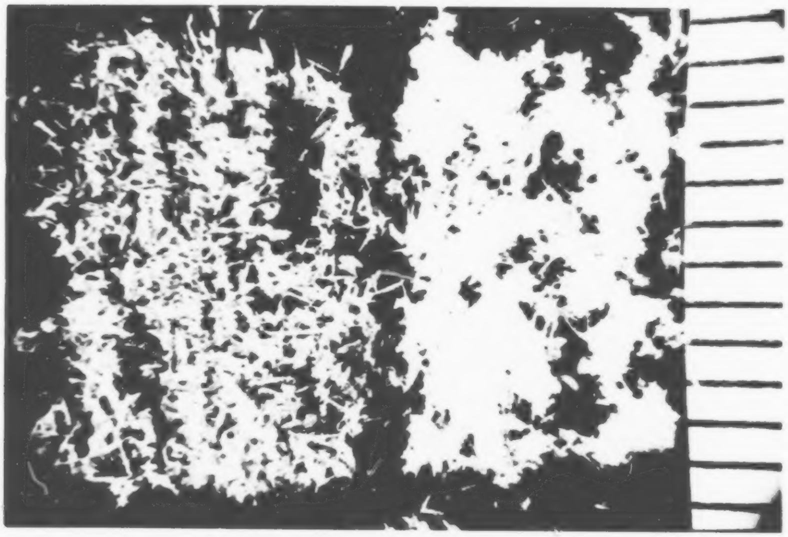


1. Douglas fir bark, extruded using laboratory model blocking machine.
2. Douglas fir bark fireplace logs, Glomera-American Company.
3. Western hemlock bark log, Oregon Timber Products Company.
4. Douglas fir + western hemlock bark fireplace log, Oregon Timber Products Company.

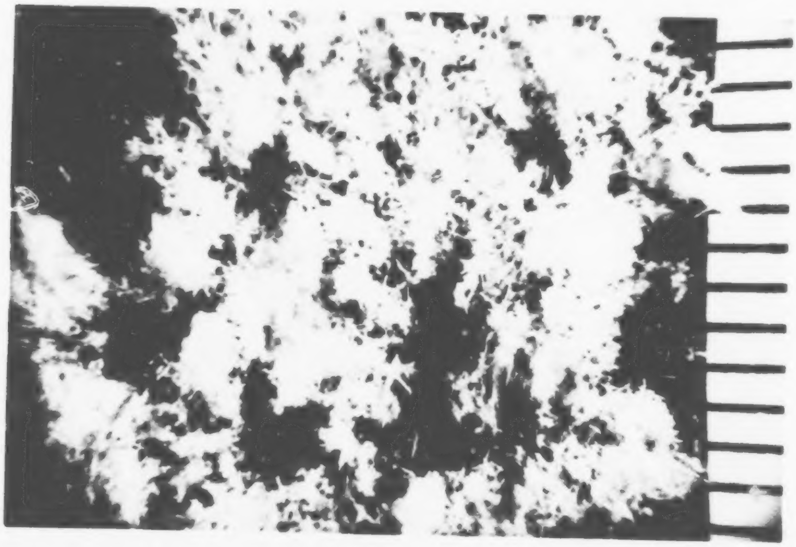


1. Injection molded switchboxes, 50% polyethylene and 50% western hemlock bark.
2. Injection molded traffic button, 50% cellulose butyrate and 50% western hemlock bark.
3. Sofa leg injection molded from 40% cellulose butyrate and 60% western hemlock.
4. Extruded shape from 70% polyethylene and 30% Douglas fir bark.

Figure 3

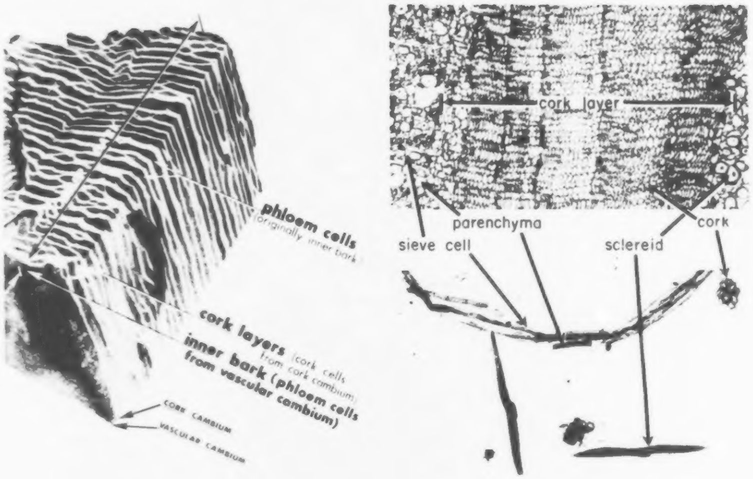


Carbohydrate fraction from Douglas fir inner bark. Left side: Inner bark starting material, fibrous in nature. Right side: Carbohydrate (holocellulose) fraction, 40-50% yield, pure white, still fibrous in nature.



Carbohydrate (holocellulose) fraction from inner bark at a higher magnification than above. It shows the white, fibrous nature of the material.

Figure 4


phloem cells
(originally inner bark)
cork layers (cork cells from cork cambium)
inner bark (phloem cells from vascular cambium)
CORK CAMBIUM
VASCULAR CAMBIUM
cork layer
parenchyma
cork
sieve cell
sclereid

Anatomy of Douglas fir Bark

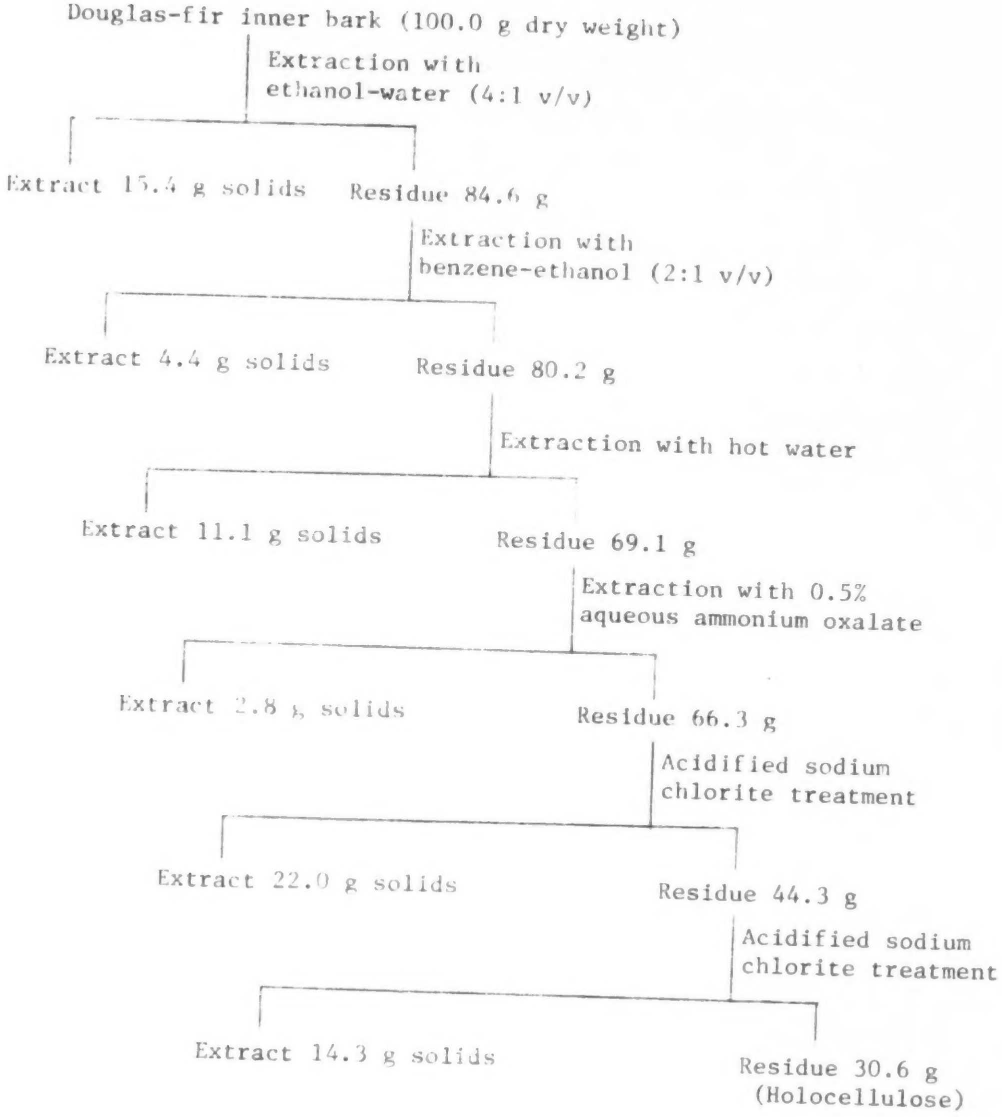

Douglas-fir inner bark (100.0 g dry weight)
Extraction with ethanol-water (4:1 v/v)
Extract 15.4 g solids
Residue 84.6 g
Extraction with benzene-ethanol (2:1 v/v)
Extract 4.4 g solids
Residue 80.2 g
Extraction with hot water
Extract 11.1 g solids
Residue 69.1 g
Extraction with 0.5% aqueous ammonium oxalate
Extract 2.8 g solids
Residue 66.3 g
Acidified sodium chlorite treatment
Extract 22.0 g solids
Residue 44.3 g
Acidified sodium chlorite treatment
Extract 14.3 g solids
Residue 30.6 g (Holocellulose)

Figure 5. Isolation of the holocellulose fraction of Douglas-fir inner bark, (g/100 g dry-weight basis).

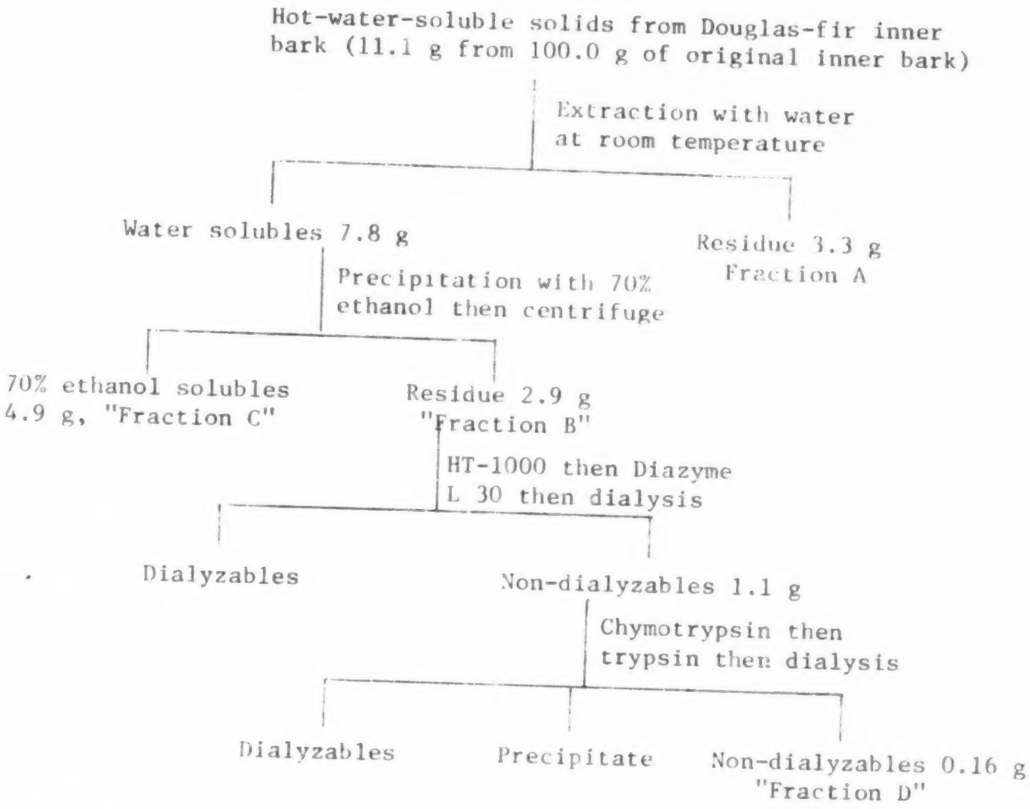

Hot-water-soluble solids from Douglas-fir inner bark (11.1 g from 100.0 g of original inner bark)
Extraction with water at room temperature
Water solubles 7.8 g
Residue 3.3 g Fraction A
Precipitation with 70% ethanol then centrifuge
70% ethanol solubles 4.9 g, "Fraction C"
Residue 2.9 g "Fraction B"
HT-1000 then Diazyme L 30 then dialysis
Dialyzables
Non-dialyzables 1.1 g
Chymotrypsin then trypsin then dialysis
Dialyzables
Precipitate
Non-dialyzables 0.16 g "Fraction D"

Figure 6. Purification of the polysaccharides extracted by hot water.

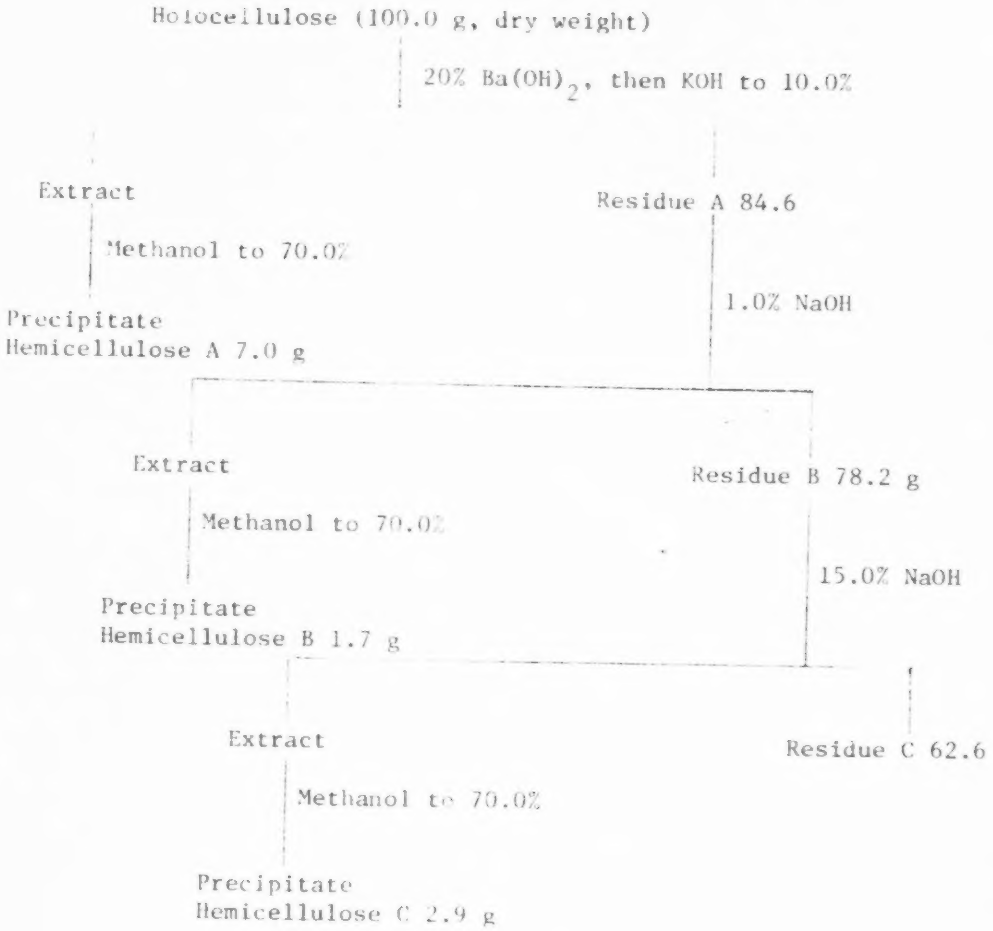

Holocellulose (100.0 g, dry weight)
20% Ba(OH)2, then KOH to 10.0%
Extract
Methanol to 70.0%
Precipitate
Hemicellulose A 7.0 g
Residue A 84.6
1.0% NaOH
Extract
Methanol to 70.0%
Precipitate
Hemicellulose B 1.7 g
Residue B 78.2 g
15.0% NaOH
Extract
Methanol to 70.0%
Precipitate
Hemicellulose C 2.9 g
Residue C 62.6

Figure 7. Fractionation of the holocellulose from Douglas-fir inner bark into its component hemicelluloses.